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BUBACAR EMBALÓ

AVALIAÇÃO DE DOIS SISTEMAS DE CLASSIFICAÇÃO DAS DISPLASIAS EPITELIAIS ORAIS E ASSOCIAÇÃO COM FATORES DE PROGNÓSTICO

FLORIANÓPOLIS/SC
2018

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Dissertação submetida ao Programa de Pós-Graduação em Odontologia, Centro de Ciências da Saúde, da Universidade Federal de Santa Catarina como requisito parcial para obtenção do título de Mestre em Odontologia - Área de Concentração: Diagnóstico Bucal.
Orientadora: Profª. Dra. Elena Riet Correa Rivero
Coorientador: Prof Dr. Filipe Modolo Siqueira

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Esta dissertação foi julgada adequada para obtenção do Título de "Mestre em Odontologia- Área de concentração Diagnóstico Bucal", e aprovada em sua forma final pelo Programa de Pós-Graduação em Odontologia.

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RESUMO

A displasia epitelial oral (DEO) consiste em alterações histológicas caracterizadas pela presença de células atípicas em vários graus de anormalidade e representa o fenótipo morfológico dos diferentes passos na progressão de um tecido normal a neoplásico. Os sistemas de classificação das DEOs são subjetivos e apresentam baixa reprodutibilidade. O objetivo do presente estudo foi avaliar a reprodutibilidade de dois sistemas de classificação das displasias epiteliais orais e correlacioná-los com as expressões das proteínas ki-67 e p53 e fatores de prognóstico (aparência clínica da lesão, localização da lesão, tabaco, álcool, sexo e idade) das Lesões Orais Potencialmente Malignas (LOPM). Foram incluídos neste estudo 66 casos de displasias epiteliais orais, os quais foram classificados segundo a Organização Mundial da Saúde (OMS) em displasias leves, moderadas ou severas, e segundo o sistema binário em displasias de baixo risco ou alto risco de transformação maligna. Todos os casos foram fotografados e analisados duas vezes, com intervalo de 7 dias, por dois avaliadores previamente calibrados. Casos em que houve discordância no diagnóstico final entre os dois avaliadores, foram avaliados por um terceiro avaliador. A variabilidade inter e intra-observador foi verificada por meio do coeficiente de Kappa. Para associar os dois sistemas de classificação das displasias, expressão dos marcadores imunoistoquímicos e com os fatores de risco foi utilizado o teste não paramétrico de Kruskal Wallis e modelo de regressão logística. O sistema binário apresentou maior concordância intra e inter-observador em relação a classificação da OMS (valor kappa 0,61 e 0,58, respectivamente). Os fatores de risco: localização em língua e lesões não homogêneas parecem estar mais associadas com as displasias de alto risco de transformação maligna classificadas pelo sistema binário. A expressão imunoistoquímica de Ki-67 e p53 aumentou progressivamente de acordo com o grau da displasia, entretanto, apenas o sistema binário foi estatisticamente significativo (p=0,012 e p=0,037, respectivamente). O sistema binário demonstrou maior reprodutibilidade, associação com as proteínas Ki-67, p53 e alguns fatores de riscos.

Palavras-chave: Leucoplasia. Proliferação celular. Proteína supressora de tumor p53. Câncer oral.

ABSTRACT

Oral epithelial dysplasia (OED) consists of histological changes characterized by the presence of atypical cells at various degrees of abnormality and represents the morphological phenotype of the different steps in the progression of a normal to neoplastic tissue. The classification systems of OEDs are subjective and have low reproducibility. The aim of the study is to assess the reproducibility of two classification systems for oral epithelial dysplasia and to correlate them with the expressions of ki-67 and p53 proteins and prognostic factors (clinical appearance of the lesion, site of lesion, tobacco, alcohol, sex and age) of Potentially Malignant Oral Lesions (PMOL).The study included 66 cases of oral epithelial dysplasia, which were classified according to the World Health Organization (WHO) in mild, moderate or severe dysplasia, and according to the binary system in low-risk or high risk of malignant transformation. All cases were photographed and analyzed twice, with a 7-day interval, by two previously calibrated raters. Cases where there was disagreement in the final diagnosis between the two observers were evaluated by a third observer. Inter- and intra-observer variability was assessed using the Kappa coefficient. The non-parametric Kruskal Wallis test and logistic regression model were used to associate the two classification systems of dysplasia with of the immunohistochemical labeling and with the risk factors. The binary system presented superior intra and interobserver agreement than to WHO classification (kappa value 0.61 and 0.58, respectively). Risk factors: tongue lesions and non-homogeneous seem to be more associated with binary system. The immunohistochemical expression of Ki-67 and p53 increased progressively according to the degree of dysplasia, however, only the binary system was statistically significant ($p = 0.012$ and $p = 0.037$, respectively). The binary system demonstrated high reproducibility, associated with risk factors and Ki-67 and p53 protein expression.

Keywords: Leukoplakia. Cell proliferation. Tumor suppressor protein p53. Mouth neoplasms.

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LISTA DE ABREVIATURAS E SIGLAS

ATPS.....3-aminopropyltriethoxysilene

DAB.....Diaminobenzidina

DARM.....Displasia alto risco de malignização

DBRM.....Displasia baixo risco de malignização

DEL.....Displasia epitelial leve

DEO.....Displasias epiteliais orais

DEM.....Displasia epitelial moderada

DES.....Displasia epitelial severa

H&E.....Hematoxilina e Eosina

H2O2.....Peróxido de hidrogênio

INCA.....Instituto Nacional de Câncer

Ki-67.....Proteína ki-67

LOPM.....Lesões orais potencialmente malignas

LPB.....Laboratório de patologia bucal

OMS.....Organização Mundial da Saúde

p53.....Proteína p53

PBS.....Tampão Fosfato-salino

UFSC.....Universidade Federal de Santa Catarina

SUMÁRIO

1 INTRODUÇÃO

O câncer é responsável por mais de 12% de todas as causas de óbito no mundo, ou seja, mais de 8 milhões de pessoas morrem anualmente da doença (FORMAN; FERLAY, 2014). Esta doença constitui o principal problema de saúde pública dos países desenvolvidos, bem como daqueles em desenvolvimento. O Brasil vem acompanhando o perfil epidemiológico mundial do câncer e adaptando-se as novas caraterísticas sócio demográficas, novo estilo de vida e intensa exposição aos fatores de risco contemporâneos (INCA, 2018). Para o biênio 2018-2019, estima-se a ocorrência de cerca 11.200 casos novos de câncer de boca em homens e 3.500 em mulheres (INCA, 2018).

A etiologia do câncer de boca é multifatorial. Os principais fatores de risco são o tabaco e o álcool, porém fatores dietéticos, infecções de cavidade oral e predisposição genética podem estar associados ao câncer de boca (HIRSHBERG *et al.,* 2014; RIVERA, 2015). Há consenso na literatura que pacientes de gênero feminino, idade maior que 45, lesões não homogêneas ou vermelhas, localizadas em língua ou assoalho de boca, com tamanho maior que 200mm2 e portadoras de displasia severa ou alto risco de transformação maligna possuem risco aumentado de transformação maligna (DOST *et al.,* 2013; SPEIGHT; KHURRAM; KUJAN, 2018).

O desenvolvimento do câncer requer múltiplas etapas que ocorrem ao longo de muitos anos (PATTON, 2018). O câncer de boca, de modo geral, é precedido por alterações da mucosa, que constituem diferentes lesões, tais como leucoplasia, eritroplasia, líquen plano, fibrose submucosa e queilite actínica, consideradas, segundo Organização Mundial de Saúde (OMS), como lesões orais potencialmente malignas (LOPMs) (REIBEL *et al.,* 2017; SPEIGHT; KHURRAM; KUJAN, 2018). Em uma revisão sistemática os autores apontam a prevalência global das lesões orais potencialmente malignas (LOPMs) para 4,7% (MELLO *et al.,* 2018).

As LOPMs podem apresentar diversos aspectos histopatológicos: hiperqueratose, acantose e grau variado de displasia epitelial (NAPIER; SPEIGHT, 2008). As displasias epiteliais orais apresentam alterações arquiteturais e citológicas do epitélio causadas pelo acumulo de alterações genéticas e associadas a um risco aumentado de progressão para o carcinoma epidermoide (REIBEL *et al.,* 2017). Portanto, é necessária a detecção, acompanhamento e diagnóstico precoce das

LOPMs, com indispensável avaliação histopatológica para viabilizar o gerenciamento clínico e evitar supostas progressões malignas.

A OMS, baseando-se essencialmente nas características citológicas e arquiteturais, propôs a classificação das displasias epiteliais orais (DEOs), em leve, moderada e severa, com o intuito de facilitar o diagnóstico e prognóstico das LOPMs (REIBEL *et al.*, 2017). Tal sistema é considerado subjetivo e carece de reprodutibilidade intra e inter-observador, devido à insuficiência de critérios morfológicos validados e a natureza biológica das displasias (KUJAN *et al.*, 2006). Nesse contexto, Kujan *et al.*, (2006), criaram o sistema binário, onde as displasias epiteliais são classificadas em alto risco e baixo risco de malignização , posteriormente aceito pela OMS (REIBEL *et al.*, 2017).

Estudos sugerem o uso de marcadores biológicos moleculares como complemento do diagnóstico histopatológico na avaliação prognóstica das LOPMs e também, para reduzir a grande variabilidade e baixa reprodutibilidade dos sistemas de classificação (CALDEIRA; ABREU; CARMO, 2012; REIBEL, 2003). O Ki-67 e p53 são amplamente utilizados como indicadores de progressão e agressividade tumoral (HUMAYUN; PRASAD, 2011). O Ki-67 é um marcador nuclear de proliferação celular presente em todas as fases (G1, S, G2 e mitose) do ciclo e divisão celular, exceto G0 (SCHOLZEN; GERDES, 2000). A imunomarcação de Ki-67 pode distinguir as LOPMs, estimando o índice de proliferação (SHAILAJA *et al.*, 2015). O p53 é um gene supressor do tumor, que tem um papel importante na carcinogênese (SABAPATHY; LANE, 2017). A mutação do p53 contribui para a complexa rede de eventos moleculares que levam a malignização (ZHANG *et al.*, 2017), estando presente em 35% de carcinomas epidermoides de boca (SOARES *et al.*, 2006). Deste modo, entende-se que a associação da imunomarcação para Ki-67 e p53 com o grau de displasia epitelial, pode melhorar a predição na progressão das LOPMs para o carcinoma epidermoide.

Desta forma, o objetivo do presente estudo foi analisar, reproduzir e comparar os dois sistemas de classificação das displasias epiteliais orais e correlacioná-los com as expressões das proteínas ki-67 e p53 e fatores de prognóstico das LOPMs que apresentam diversos graus de displasia epitelial.

1.1 JUSTIFICATIVA

As lesões potencialmente malignas apresentam alta taxa de transformação maligna. Atualmente, o diagnóstico, prognóstico e conduta clínica frente as LOPMs dependem da gradação histopatológica das displasias epiteliais, que é baseada em classificações subjetivas e de baixa reprodutibilidade. Os dados relatados na literatura quanto à relação de grau da displasia e a progressão das LOPMs são imprecisos, devido à inviabilidade ética de realizar estudos prospectivos que visem avaliar e acompanhar tais lesões quanto a sua progressão para carcinoma epidermoide.

Portanto, há necessidade de validar, reproduzir e comparar os dois sistemas de classificação de forma independente e verificar qual dos dois sistemas está melhor relacionado com o risco de progressão das lesões orais potencialmente malignas.

1.2 PERGUNTA NORTEADORA

Qual sistema de classificação das displasias epiteliais orais tem maior reprodutibilidade e está relacionado com os fatores de risco de evolução para carcinoma epidermoide de boca?

2 REVISÃO DE LITERATURA

2.1 LESÕES ORAIS POTENCIALMENTE MALIGNAS

A leucoplasia, eritroplasia, queilite actínica, líquen plano e fibrose submucosa são consideradas, segundo a OMS, LOPMs (REIBEL *et al.,* 2017). Essas lesões, quando não tratadas podem evoluir para câncer de boca (WARNAKULASURIYA; ARIYAWARDANA, 2016). Neste estudo, somente as leucoplasias, eritroplasias e eritroleucoplasias foram abordadas.

A leucoplasia é a LOPM mais frequente na mucosa oral e é definida, segundo Reibel *et al.,* (2017) como uma placa branca, não removível a raspagem e que não pode ser classificada clinicamente ou patologicamente como outra lesão específica (REIBEL *et al.,* 2017). Pesquisas apontam o tabaco, álcool, exposição solar, diabetes e infecções da cavidade oral como possíveis fatores etiológicos para a leucoplasia (REIBEL *et al.,* 2017; RIVERA, 2015) que normalmente acomete homens de meia idade, idosos e tabagistas crônicos (WARNAKULASURIYA, 2018).

O potencial de malignização das leucoplasias gera divergências entre pesquisadores. Wang *et al.,* (2014) acompanharam 5071 pacientes com lesões potencialmente malignas e observaram uma taxa de transformação de 4,32% (WANG *et al.,* 2014). Mohammed & Fairozekhan (2017), apresentaram uma prevalência global de leucoplasia de 2,6% e taxa de transformação maligna variando de 0,1% a 17,5%, enquanto que Reibel *et al.,* (2017) estimaram a taxa global média de transformação maligna de leucoplasia em 1-2% (MOHAMMED; FAIROZEKHAN, 2017; REIBEL *et al.,* 2017). Lesões em língua e assoalho bucal possuem maiores taxas de transformação maligna para carcinoma (SPEIGHT; KHURRAM; KUJAN, 2018).

Na avaliação histológica, a leucoplasia pode apresentar diversos aspectos, como hiperceratose, acantose, atrofia do epitélio, e vários graus de displasia epitelial (MAIA *et al.,* 2016). Portanto, o exame histopatológico é indispensável para determinar o prognostico clínico e terapêutico destas lesões.

A eritroplasia é definida como uma placa ou mancha vermelha que não pode ser classificada clinicamente como qualquer outra entidade patológica (WARNAKULASURIYA, 2018). A causa de eritroplasia é desconhecida, porém, tabaco e álcool são considerados fatores etiológicos importantes (WARNAKULASURIYA, 2018). A eritroplasia afeta predominantemente homens entre 65 a 74 anos de

idade. Clinicamente, apresenta-se como uma mácula ou placa eritematosa bem demarcada com uma textura macia e aveludada, e é encontrada comumente na língua, assoalho de boca e palato mole unilateralmente (SILVEIRA *et al.*, 2009; VAN DER WAAL, 2014). A prevalência de eritroplasia varia de 0,2% a 0.83%(REICHART; PHILIPSEN, 2005) . O potencial de transformação maligna da eritroplasia varia de 14% a 50%, porém, mais de 90% dos casos apresentam displasia moderada ou severa (SILVEIRA *et al.*, 2009). No entanto, Mello *et al.*, (2018) evidenciaram que apesar da baixa prevalência da eritroplasia entre as LOPMs, a prevalência de displasia epitelial severa e carcinoma in situ na análise histopatológica das lesões vermelhas foi maior do que na análise histopatológica de lesões brancas (MELLO *et al.*, 2018).

A eritroplasia quando associada a leucoplasia é denominada de eritroleucoplasia (AWADALLAH *et al.*, 2018). Vale salientar que dentre as lesões orais potencialmente malignas, as lesões vermelhas e não homogêneas apresentam fatores de risco de maior importância para transformação maligna (AWADALLAH *et al.*, 2018; SPEIGHT; KHURRAM; KUJAN, 2018).

2.2 DISPLASIAS EPITELIAIS ORAIS

A displasia epitelial consiste em alterações histológicas caracterizadas pela presença de células atípicas em vários graus de anormalidade e representa o fenótipo morfológico dos diferentes passos na progressão de um tecido normal a neoplásico (CURY *et al.*, 2007).

A displasia é um diagnóstico definido pela presença das características citológicas e arquiteturais do epitélio alteradas (Tabela 1). O que significa que uma mucosa com displasia epitelial tem um risco aumentado de evoluir para carcinoma quando comparada à mucosa normal (WARNAKULASURIYA *et al.*, 2008).

Desde 1978, vários sistemas de classificação das displasias epiteliais foram criados com a finalidade de facilitar a troca de informação entre os patologistas, acompanhar a evolução das intervenções, comparar as respostas dos tratamentos e traçar o prognóstico das DEOs (PINDBORG; REIBEL; HOLMSTRUP, 1985). Atualmente, embora com características bem conhecidas, a classificação das DEOs continua sendo subjetiva, demonstrando baixa reprodutibilidade e pouca relação com a transformação maligna (KUJAN *et al.*, 2006; NANKIVELL *et al.*, 2013).

A organização mundial de saúde (OMS) no seu último relatório recomendou o uso do sistema binário e também, o sistema de classificação segundo OMS (REIBEL *et al.*, 2017). Em ambos os sistemas as lesões são classificadas com base nas características citológicas e arquiteturais (tabela 1).

Para a classificação segundo a OMS, as displasias epiteliais orais são graduadas em três níveis (REIBEL *et al.* 2017):

1. **Displasia epitelial leve:** é caracterizada pela presença de alterações celulares e arquiteturais restritas ao terço basal do epitélio;
2. **Displasia epitelial moderada:** quando as alterações se estendem até o terço médio do epitélio afetado;
3. **Displasia epitelial severa:** quando as alterações ultrapassam o terço médio e estendem-se pelo terço superior. Por fim, quando a lesão apresenta displasias em abundância e abrangendo todos os terços do epitélio, passa a ser diagnosticada como **carcinoma *in situ.***

Tabela 1Critérios citológicos e arquiteturais das displasias epiteliais orais

Critérios Citológicos	Critérios Arquiteturais
Variação no tamanho do núcleo	Estratificação epitelial irregular
Variação no formato do núcleo	Alteração da polaridade das células basais
Variação no tamanho da célula	Cristas epiteliais em forma de gota
Variação no formato da célula	Pérolas de ceratina
Aumento no tamanho do núcleo	Número aumentando de figuras mitóticas
Proporção núcleo/ citoplasma alterado	Mitoses superficiais anormais
Figuras mitóticas atípicas	Disceratose
Aumento no número e tamanho dos nucléolos	
Hipercromatismo nuclear	

Estudos apontam baixa reprodutibilidade inter-observador variando de concordância fraca a moderada (KUJAN *et al.*, 2007; NANKIVELL *et al.*, 2013; KRISHNAN *et al.*, 2016). Os problemas na avaliação da displasia epitelial decorrem da falta de objetividade na avaliação dos critérios estabelecidos, divisão arbitrária das graduações, falta de calibração dos critérios de graduação, e falta de conhecimento suficiente de quais critérios são importantes para a predição de potencial maligno (WARNAKULASURIYA *et al.*, 2011)

O sistema binário de classificação foi proposto por Kujan *et al.*, (2006), com intuito de tornar a classificação das displasias epiteliais

mais objetivo, quantificável, reprodutível, com menor variabilidade, e melhor capacidade prognóstica.

O sistema binário de classificação agrupa as displasias epiteliais orais em dois grupos:

1.**Displasia epitelial de baixo risco de malignização:** quando presente, menos de quatro critérios arquiteturais e cinco critérios citológicos no epitélio.

2.**Displasias epiteliais de alto risco de malignização**: quando presente, no mínimo, quatro critérios arquiteturais e cinco critérios citológicos no epitélio.

Kujan *et al.* (2006), avaliaram a reprodutibilidade e a precisão do sistema binário, comparando-o com o sistema de classificação segundo a OMS. Os resultados alcançados foram satisfatórios quanto à distinção da displasia leve, severa e carcinoma *in situ*, usando a classificação da OMS, porém, a avaliação da displasia moderada mostrou-se incerta. Em contraste com a classificação da OMS, o sistema binário apresentou alta precisão na classificação das displasias (80%), alta sensibilidade (85%), alta especificidade (80%), maior consenso entre os patologistas e estatisticamente significante quando associado com as características clinicas das lesões (KUJAN *et al.*, 2006)

Ainda, Kujan *et al.*, (2007), na tentativa de compreender os motivos da variabilidade na classificação das DEOs, avaliaram individualmente cada critério morfológico conforme proposto pela OMS (2005) e em seguida, estes critérios foram associados com as características clínicas das lesões. Os autores, evidenciaram maior concordância inter- examinador para os seguintes critérios: aumento do tamanho de figuras mitóticas, cristas epiteliais em forma de gota, aumento do tamanho nuclear e variação anormal na forma da célula (KUJAN *et al.*, 2007). Os maiores índices de desacordo na concordância foram observados nos seguintes critérios: estratificação epitelial irregular, perda de polaridade das células basais, variações anormais no tamanho nuclear, figuras mitóticas atípicas e hipercromatismo celular, porém, houve um acordo bom a substancial quanto a classificação das displasias epiteliais em alto e baixo risco (KUJAN *et al.*, 2007). Quanto a associação dos critérios arquiteturais e citológicos com as características clínicas, os autores observaram que, a perda de polaridade de células basais, cristas epiteliais em forma de gota, número aumentado de figuras mitóticas, variação anormal da forma do núcleo, variação anormal do tamanho e forma das células, mitoses atípicas, aumento do número e tamanho dos nucléolos foram as únicas

características estatisticamente significantes, associadas com os resultados clínicos (KUJAN *et al.*, 2007).

Nankivell *et al.*, (2013) e Krishnan *et al.*, (2016), apresentaram resultados similares em comparação com Kujan *et al.* (2006 e 2007) quanto a consistência do sistema binário na classificação das displasias epiteliais e resultados conflitantes na avaliação individual das características citológicas e arquiteturais .

2.3 PROTEÍNA Ki-67

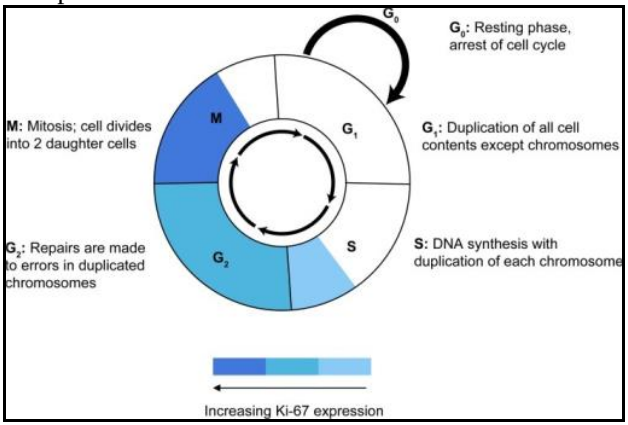
As LOPMs apresentam um amplo espectro de alterações histológicas, por isso, há necessidade do uso de marcadores biológicos como complemento à classificação das DEOs, principalmente por participarem ativamente na carcinogênese oral (IATROPOULOS & WILLIAMS, 1996).

O antígeno Ki-67, foi originalmente identificado por Scholzer e Gerdes na década de 80 (SCHOLZEN & GERDES, 2000) e está presente em todas as fases do ciclo celular (G1, S, G2 e M) e ausente nas células quiescentes (G0), possuindo meia-vida de aproximadamente 1-1,5h (figura1) (SCHOLZEN & GERDES, 2000; LI *et al.*, 2015). A proliferação celular é um processo circadiano, estimulado por processos que incluem principalmente substancias antigênicas, isquemia do tecido, citotoxicidade e morte celular (IATROPOULOS & WILLIAMS, 1996).

O índice de proliferação de Ki-67 tem sido usado não somente para distinguir o comportamento biológico das lesões malignas e potencialmente malignas, mas também para prever o comportamento clinico/biológico destas lesões (RODRIGO *et al.*, 2012).

Angiero *et al.*, (2008), investigaram a expressão das proteínas p16, p53 e Ki-67 em 25 DEOs, 11 carcinomas e 18 casos sem displasia. Os autores observaram que os marcadores foram expressos apenas na camada basal dos casos sem displasia e displasia leve, e nas demais displasias observaram aumento progressivo de imunopositividade de acordo com o grau de displasia de cada caso . Birajdar et al. (2014), observaram que a imunoexpressão de Ki-67 é proporcional ao grau histológico, principalmente, devido ao aumento da atividade proliferativa celular .

Figura 1Expressão do Ki-67 durante as fases do ciclo celular.



Fonte:https://openi.nlm.nih.gov/detailedresult.php?img=PMC4385855_oncotarget-06-2331-g006&req=4

2.4 PROTEÍNA p53

A proteína p53 é codificada pelo gene supressor de tumores (TP53), localizado no braço curto de cromossomo 17. É considerada uma importante proteína no mecanismo de autorregulação das células neoplásicas. A p53 se liga a sequências específicas de DNA a fim de controlar, de forma positiva ou negativa, a expressão de diversos genes envolvidos no controle do ciclo celular (SUPOWIT, 1984). Devido a esta característica, foi chamado de "Guardião do genoma" (LANE, 1992).

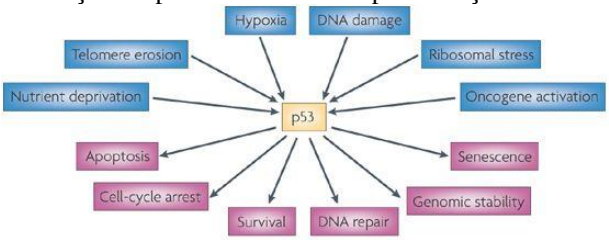
A p53 regula a atividade de vias de sinalização que levam a uma variedade de interrupções do ciclo celular, incluindo hipóxia, ativação da oncogênese, biogênese mitocondrial e ribossômica alterada e manutenção de centrossoma. Dependendo do nível de comprometimento celular, a p53 pode induzir morte celular, senescência ou parada do ciclo celular e posterior reparo do DNA (figura2) (SABAPATHY; LANE, 2017).

Segundo o *National Center for Biotechnology Information* (US), (1998), a proteína p53 se liga ao DNA, que por sua vez estimula a produção do p21, que interage com cdk2 (proteína estimuladora da divisão celular). Quando há anormalidades no complexo p21 e cdk2, a divisão celular fica comprometida. Então, efetivamente, o p53 perde o

vínculo com o DNA e consequentemente perde a função, levando a um aumento da instabilidade genômica. Assim, as células se dividem e replicam incontrolavelmente, com mutações genéticas adicionais que podem impulsionar a progressão e desenvolvimento de neoplasias malignas (NCBI, 1998).

Atualmente, a p53 é amplamente estudada em neoplasias malignas e em lesões orais potencialmente malignas. A mutação do gene p53 é encontrada em mais de 35% de carcinomas orais (SOARES *et al.*, 2006). A marcação de p53 é nuclear e o índice de expressão tende a aumentar de acordo com o grau da displasia (CRUZ *et al.*, 2002). Estudos mostram que a marcação de Ki-67 e p53 acima da camada suprabasal ou com porcentagem de células positivas maior que 25%, é indicativo de alto risco para progressão maligna (KANNAN *et al.*, 1996; CRUZ *et al.*, 1998; HUMAYUN & PRASAD, 2011)

Figura 2Função do p53 no controle de proliferação celular.



Fonte: Lane (2007)

2.5 FATORES DE RISCO PARA PROGRESSÃO MALIGNA

Os principais fatores de risco para progressão das lesões orais potencialmente malignas são: idade, gênero, hábitos de fumar, consumo de álcool, características clínicas da lesão (localização anatômica da lesão, cor e textura, tamanho e extensão), presença de displasia epitelial e superexpressão das proteínas Ki-67 e p53(CRUZ *et al.*, 2002; SPEIGHT; WARNAKULASURIYA; ARIYAWARDANA, 2016; KHURRAM; KUJAN, 2018). Identificar os fatores de risco nos pacientes portadores de LOPMs pode ajudar na prevenção de progressão para carcinoma.

Autores apontam que a prevalência de câncer de boca aumenta de acordo com a idade, já que indivíduos jovens possuem menor risco e tempo de exposição a agentes cancerígenos (CHUNG et al. 2005; KUMAR *et al.*, 2015).

O tabaco contém vários elementos químicos, considerados pré-cancerígenos e basicamente, podem ser agrupados em três grupos: nitrosaminas, benzopirenos e aminas aromáticas (H.MONTERO & G. PATEL, 2015; KUMAR *et al.,* 2016). De acordo com Rivera, (2015), o tabaco expõe o epitélio oral aos radicais livres de oxigênio e nitrogênio, que podem afetar mecanismos de defesa e promover mutações no DNA (RIVERA, 2015). O álcool pode atuar como um fator de risco local e sistêmico, aumentando a permeabilidade da mucosa oral e dissolução de componentes lipídicos do epitélio, podendo acarretar em atrofia epitelial (RIVERA, 2015). Tabaco e álcool juntos possuem um efeito sinérgico e a sua exposição prolongada, pode causar anormalidades genéticas e comprometimento do reparo do DNA (RAM *et al.,* 2011; RIVERA, 2015).

As LOPMs podem acometer qualquer sítio na cavidade oral (MONTERO; G. PATEL, 2015). A língua (borda lateral e ventre), assoalho da boca, rebordo alveolar, gengiva, mucosa jugal, mucosa labial e palato, são os sítios mais acometidos. A localização da lesão pode ser crucial para progressão das LOPMs para carcinoma (NARAYAN & SHILPASHREE, 2016). Napier *et al.,* (2003), mostraram que a borda lateral da língua, ventre da língua e assoalho da boca, são localizações com maiores taxas de transformação maligna (NAPIER *et al.,* 2003).

Autores relatam que lesões com características clínicas não homogêneas, vermelhas e com tamanho e extensão maior que 200mm2, possuem risco aumentado de progressão para carcinoma (ARDUINO *et al.,* 2009; DOST *et al.,* 2013). Também, apresentam maior prevalência de alteração do gene p53, comparado com as lesões homogêneas e brancas (BIRAJDAR *et al.,* 2014).

Napier *et al.,* (2003), observaram associação estatística significativa entre as aparências clínicas e a extensão das LOPMs com o desenvolvimento de câncer de boca. Ho *et al.,* (2012), avaliaram três parâmetros clínicos: transformação maligna, progressão do grau de displasia e remissão ou estabilidade da displasia em 91 pacientes e relataram taxa de progressão maligna de 22% aos 5 anos. De acordo com os autores, alto grau de displasia, lesões localizadas na assolho de boca e borda lateral da língua, tamanho >200 mm2, lesões não homogêneas e tabagismo, foram fatores fortemente associados com a transformação das LOPMs para câncer (HO *et al.,* 2012).

Estudos mostraram que a presença de displasia é um fator de risco preeminente para progressão das LOPMs (MEHANNA *et al.,* 2009; LIU *et al.,* 2010; WARNAKULASURIYA *et al.,* 2011). Porém,

estabelecer a taxa de transformação maligna baseando-se somente no grau de displasia é incerto, devido a sua alta subjetividade e associação com os fatores de risco acima citados (KUJAN *et al.*, 2006).

As LOPMs apresentam alta taxa de transformação maligna. Sabe-se que os sistemas de classificação das displasias epiteliais orais possuem alta subjetividade e os critérios morfológicos são de difícil reprodução. A associação dos fatores de risco com as proteínas ki-67 e p53 e os sistemas de classificação das DEOs podem contribuir para predição na progressão das LOPMs para carcinoma. Desta forma, o presente estudo visa contribuir para a validação dos sistemas de classificação das DEOs e fornecer evidências na literatura que possam auxiliar o patologista na rotina diária do diagnóstico histopatológico, principalmente em relação aos casos críticos que podem levantar dúvidas e controvérsias entre patologistas.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar a reprodutibilidade de dois sistemas de classificação das displasias epiteliais orais e associá-los com os fatores de risco para o desenvolvimento do carcinoma epidermoide de boca.

3.2 OBJETIVOS ESPECÍFICOS

Verificar a reprodutibilidade intra e inter-observador na classificação da OMS e na classificação binária das displasias epiteliais;

Avaliar no sistema binário quais os critérios de pior e melhor reprodutibilidade;

Avaliar os dois sistemas de classificação das displasias em relação à expressão de proteínas relacionadas com o risco de progressão das displasias epiteliais, dentre estas o marcador de proliferação Ki-67 e a proteína p53;

Avaliar a relação de cada sistema de classificação com os fatores de risco clínicos para o câncer de boca.

4 ARTIGO

Artigo formatado conforme as normas da revista Oral Oncology (acessadas em: 09/06/2018).

EVALUATION OF TWO SYSTEMS OF CLASSIFICATION OF ORAL EPITHELIAL DISPLASIA AND ASSOCIATION WITH PROGNOSTIC FACTORS

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ABSTRACT

Objectives: The aim of this study was to evaluate the inter- and intra-observer reproducibility of oral epithelial dysplasia (OED) classification system according to WHO and binary system and to associate them with imunoexpression of Ki-67 and p53, as well as clinical risk factors. Materials and Methods: Sixty - six cases were evaluated using the two classification systems. The inter- and intra-observer variability and prognostic capability of both systems were evaluated using Kappa and logistic regression model, respectively. The immunohistochemistry was carried out to assessed the expression of Ki-67 and p53. Results: The binary system showed higher agreement than to the WHO classification (kappa value 0.61 and 0.58, respectively). The binary system seems to be associated with risk factors such as tongue lesions (OR = 4.32, p=0.008) and non homogeneous lesions (OR=9.250, p=0.008 vs OR adj=6.460, p=0.037). Protein expression increased progressively according to the degree of dysplasia, but only the binary system showed statistical significance of Ki-67 and p53 (p = 0.012 and p = 0.037, respectively). Conclusion: The binary system is more reproducible, associated with risk factors, Ki-67 and p53 protein.

Keywords: Leukoplakia; Cell Proliferation; Tumor Suppressor Protein p53.

INTRODUCTION

Oral Squamous cell carcinoma (OSCC) is situated within the top of 10 ranking incidence of cancers and despite the progress in research and therapy, survival has not improved in the last years [1]. OSCC is preceded by mucosal changes that constitute different lesions such as leukoplakia, erythroplakia and erythroleukoplakia [2,3] considered as potentially malignant oral lesions (PMOL), according to World Health Organization (WHO) [4]. The main risk factors for PMOLs progression to cancer are tobacco and alcohol consumption, lesions in the tongue, as well as non-homogenous lesions[5].

In the histopathological evaluation, these lesions may present several aspects as hyperkeratosis, acanthosis, epithelial atrophy and various degrees of oral epithelial dysplasia (OED), wich may be the most important characteristic. OED is characterized by the presence of atypical cells at varying degrees of abnormality and represents the morphological phenotype of the different stages in the progression to OSCC [6]. Studies have shown that the presence of dysplasia associated with risk factors has an increased risk for progression [7,8]. Wiht malignant transformation rates ranging from 0.13% to 36.4% in periods of 1 to 30 years [9,10].

According to the head and neck tumor classification by the WHO, epithelial dysplasia may be classified as mild (changes in the lower third of the epithelium), moderate (changes to the middle third of the epithelium), and severe (changes exceeding the middle third of the epithelium) [4]. This classification is based on analysis of cytological and architectural characteristics changes of the epithelium (Table 1) [4].

The studies indicate high subjectivity, low reproducibility, and inter- and intra-observer variability of the WHO classification system due to the arbitrary division of OED into distinct categories and lack of well-defined histological criteria [11,12]. In this context, other systems were created with objective of reducing subjectivity and increasing reproducibility in the classification of OED [11,13,14]. Among them, the binary system of classification categorizes OED into low risk (presence of at less than four architectural criteria and five cytological criteria in the epithelium) and high risk undergoing malignant transformation (presence of at least four architectural criteria and five cytological criteria)[11].

Studies suggest the use of molecular biological markers as a complement to the histopathological diagnosis in the prognostic evaluation of PMOLs and also to reduce the great variability and low

reproducibility of classification systems [15,16]. Ki-67 and p53 are used as indicators of aggression and tumor progression. Ki-67 is a nuclear marker of cell proliferation present in all phases (G1, S, G2 and mitosis) of the cycle and cell division, except G0 [17]. P53 is a tumor suppressor gene, whose mutation plays an important role in carcinogenesis and is present in 35% of OSCC [18]. The p53 mutation contributes to the complex network of molecular events leading to formation of malignancy [19]. The correlation of immunolabeling to Ki-67 and p53 with the OED may improve the prediction of the progression of PMOL to OSCC.

Our aims were to evaluate the reproducibility of the binary system and the WHO classification; To analyze inter- and intra-observer variability of each cytological and architectural criterion of the epithelium; correlate the two classification systems of OED (WHO and binary system) with the expressions of ki-67, p53 and clinical factors assessing to prognostic ability.

MATERIAL AND METHODS

This study was approved by the ethical board at Federal University of Santa Catarina (protocol number 42976715.3.0000.0121) and was carried out in compliance with the Helsinki Declaration.

Patients, lesions and samples

This was a retrospective study based on data from the archive of the Oral Pathology laboratory of the School of Dentistry, University of federal Santa Catarina. All cases clinically diagnosed as leukoplakia, erythroplakia and erythroleukoplakia diagnosed histologically as epithelial dysplasia from at the period 2007-2017 were collected. Subsequently, the cases were grouped into homogenous and non-homogenous clinical appearance. Demographics data and risk factors of the all cases were raised. Cases without a consensus diagnosis of epithelial dysplasia, cases of proliferative verrucous leukoplakia and cases adjacent to or in association with a previous diagnosis of OSCC were excluded. All cases included were classified according to the system proposed by the WHO (2017)[4] and by binary system [11].

Assessment system

For standardization purposes, all cases were photographed by a single evaluator in magnifications of 200x and 400x, in order to ensure that all dysplastic areas were evaluated. The histopathological slides and microphotographs were then randomly blinded through a labeling system, the details of which were accessible only to the non-participating assistant. Two independent observers postgraduate students (Observer B and A) in oral diagnosis evaluated the microphotographs. The microphotographs were assessed at two different periods in a minimum interval of 7 days between the 1st and 2nd evaluation for each case. All the cases in disagreement in the final classification were reassessed by the third evaluator for agreement in the final diagnosis. All evaluations were performed independently, without knowledge of clinical and histopathological data. Cases were graded with the use of the WHO system into mild, moderate or severe dysplasia. Cases were also graded with the use of the binary system into high risk (at least 4 architectural changes and 5 cytological changes) or low risk (<4 architectural changes and <5 cytological changes) after assessment of cytologic and architectural features (Table 1).

Immunohistochemistry

For the reactions were obtained 3 µm thick sections in previously silanized slides from each case. The sections were deparaffinized in xylene bath and sequentially rehydrated in ethanol, washed with distilled water and then were immersed in 6% hydrogen peroxide in methanol solution to inhibit the activity of endogenous peroxidase for 20 minutes. For antigen recovery was used 0.01 M citrate buffer (pH 6.2) in water bath for 40 min. Endogenous biotin blockage was performed by incubation of the sections in 5% skimmed milk solution in PBS for 40 minutes. Sections were incubated with primary antibodies against Ki-67 (spring Biosciences®, SP6) and p53 (Sigma Aldrich®) with a 1: 400 dilution, at 4°C overnight. Subsequently, the anti-IgG secondary antibody conjugated to a biotinylated peroxidase polymer (Envision + kit / HRP double bond rabbit/mouse, DAKO® Denmark) ready for use was applied, for one hour at room temperature. The slides were stained with DAB chromogen solution (Dako Corporation, Carpinteria, CA, USA) and counterstained with Harris hematoxylin. After dehydration and diafanization, the slides were mounted with Entellan (Merck, Darmstadt, Germany). Histopathologically confirmed case of OSCC was taken as positive control for the expression of Ki-67 and p53. A negative control for each reaction was obtained by omitting the primary antibody.

Immunohistochemical analysis

Tissue sections positive for Ki-67 and p53 were examined for the presence of brown stained nuclei regardless of intensity. Immunohistochemistry reactions were evaluated on NIH ImageJ 1.45q software (National Institutes of Health, Maryland, USA) using captured images at 400x magnification. Ki-67 and p53 immunoreactivity was defined as the mean ratio of positive nuclei among 1000 epithelial cells in up to 10 consecutive microscopic fields. Immunohistochemical analysis were assessed independently without any knowledge of the morphological findings.

Statistical analysis

Statistical analyses were performed using IBM SPSS (version 21) and MedCalc software (Version 18.5). The intra and interobserver variability of each morphological criterion (Table 1) and the binary

system was measured on an ordinal scale of two categories using the unweighted Kappa coefficient. For the WHO system we calculated the weighted kappa. The weighted Kappa is used to calculate the reproducibility when the variables are ordinal and the results expressed by more than two categories, also, gives greater importance to the discordance, assigning it greater weight in the calculation of the reproducibility. The kappa values generated were interpreted to the according standards proposed by Landis and Koch[20], with scores of 0-0.2 representing slight, 0.2-0.4 fair, 0.4-06 moderate, 0.6-0.8 substantial, and 0.8-1.0 near perfect agreement.

A logistic regression analysis was performed to evaluate the association between clinicopathological factors and the degree of epithelial dysplasia. Variables such as clinical appearance (homogeneous and non-homogeneous lesions), anatomical site (tongue, floor of the mouth, palate, buccal mucosa and retro molar), clinical risk factors (smoking and alcohol / smoking) , age (> 45 vs <45), sex (male vs. female) were added in the model to assess its association with various degrees of epithelial dysplasia categorized according to the binary system at low and high risk of malignant transformation and WHO classification in mild, moderate and severe dysplasia. The primary outcome was high-risk of malignant transformation and severe dysplasia. Data were analyzed in detail to determine their independent effect on OED prognosis in univariate models and multivariate models. For the multivariate models, only variables with univariate associations with $p \leq 0.20$ were used.

A two-way Chi-square test and Fisher exact test were used to analyze associations between variables of interest (morphological criteria, classification WHO and Binary system).

For the quantitative analysis of Ki-67 and p53, were used Kruskal-Wallis tests to compare the groups (mild, moderate and severe) and Mann-Whitney to compare low-risk and high-risk groups. The level of statistical significance of all tests was $p<0.05$.

RESULTS

Demographic characteristics and clinicopathological data included in the study are summarized in Table 2. The study included 66 patients comprising 38 females and 28 males (mean age, 56.8±12.9 years). Leukoplakia was the most common lesion involving the tongue and smokers patients. Based on the histological grading of epithelial dysplasia, proposed by WHO, our samples showed the following distribution: 35 mild, 13 moderate, and 18 severe epithelial dysplasia (Table 2). For the binary system, 27 cases were classified as high risk and 39 low risk of malignant transformation.

Immunohistochemical expression

Morphological analysis revealed that ki-67 and p53 were predominantly expressed in the basal layer in mild and moderate dysplasia (Figure 1). For severe dysplasia the labeling was predominant in the suprabasal layer. The percentage of Ki-67 and p53 was lower in cases of mild dysplasia when compared to moderate and severe dysplasia. However, the Kruskal-Wallis test did not show a statistically significant difference between the groups (mild, moderate and severe) (Figure 2). Mann-Whitney test showed a statistically significant difference between groups regarding the immunoreaction of Ki-67 and p53, as there was lower expression among low risk cases when compared with high risk cases of malignant transformation (figure 2).

Inter-and-intraobserver variability of individual architectural and cytological criteria

Our results presented great variation in the agreement of the individual cytological and architectural features (Table 3 and 4). For the observer A, abnormal variation in cell shape showed a higher agreement with Kappa value of 0.94 CI (0.83-1.00) and the Increased nuclear cytoplasmic ratio presented least agreement with kappa value of 0.34 CI (0.10-0.57). For the intra-observer assessment, observer B presented higher agreement for the irregular epithelial stratification with the kappa value of 1.0 CI (1.00-1.00) and least agreement for the increased number and size of nucleoli with a kappa value of 0.40 CI (0.14-0.65) (Tables 3 and 4). For the inter-observer assessment, abnormal variation in nuclear shape and premature keratinization in single cells showed higher agreement with kappa values of 0.54 CI (0.24-.81), 0.64 CI (0.45-0.82),

respectively. Irregular epithelial stratification and abnormal variation in nuclear shape showed lower agreement with negative kappa values, respectively of -0.03 CI (-0.08-0.02) and -0.05 CI (-0.11-0.01). However, negative Kappa value suggest that the agreement found was less than that expected by chance in the disagreement among the evaluators. Hyperchromatism was a characteristic present in all evaluated cases, however it was not analyzed due to its constancy.

Inter-and-intraobserver variability WHO classification and binary system

The results of the inter- and intra-observer assessment were presented in Table 5. For the WHO classification, the intra-examiner agreement was almost perfect (Kw = 0.93) for the observer B and substantial agreement for the observer A (k = 0.77). The WHO classification presented moderate interobserver agreement (kw = 0.58) in the first and second observation, respectively. However, the binary system showed almost perfect intra-observer agreement ranging from 1.0 C.I (1.00-1.00) to 0.87 (0.76-0.99) for the observer B and A respectively. For the interobserver assessment of the binary system, agreement was substantial for both observations.

Association of classification WHO and Binary system

The chi-square test showed a statistically significant association (p=0.001) between the two classification systems. 2.9% and 61.5% of the mild and moderate dysplasia were classified as high risk. All cases of severe dysplasia were classified as high risk of malignant transformation. In this study, the morphological criteria such as increased nuclear size, atypical mitotic figures, increased nuclear cytoplasmic ratio, irregular epithelial stratification, increased number and size of nucleoli, keratin pearls within rete ridges, increased number of mitotic figures, abnormally superficial mitoses and premature keratinization in single cells were statistically associated ($p < 0.05$) with severe dysplasia and high risk of malignant transformation.

Prognostic capability for predicting high risk dysplasia

In the present study, was performed a logistic regression model to evaluate the association between clinical factors and the presence of epithelial dysplasia. Our results demonstrated that sex, age and

smoking/alcohol consumption were not associated with OEDs in both classification systems. However, it is important to highlight that most patients with epithelial dysplasia were smokers and/or alcoholics. In the binary system, tongue lesions were 4.32-fold (95% CI=1,519-12,331; p= 0.006) more likely to have high-risk dysplasia compared to other intra oral sites, and adjusting for other variables, the odds decreases to 3.62-fold (95% C.I=1.119-11.718; p=0.032). Regarding the clinical appearance, non-homogeneous lesions were 9.25-fold (95% C.I=1.808-47.325; p=0.008) more likely to have high-risk dysplasia than homogeneous lesions.

In WHO classification, lesions with a non-homogeneous clinical appearance (p=0.007) have 10.5-fold (95% C.I=1.893-58.242) more odds to have severe dysplasia than mild dysplasia, however without statistical significance when adjusted (p = 0.182).

DISCUSSION

Despite the well-known characteristics, OED lack interobserver reproducibility and evidence high subjectivity. Our study attempted to comprehend the classification of OED and to correlate them with the expression of ki-67, p53 and clinical risk factors.

The main event that occurs in the transition from the normal oral epithelium to carcinoma is cell proliferation [21]. Mutant p53 immunoexpression is found in 90% of OPMLs and is generally absent in the normal oral mucosa. [22]. In our study, cell proliferation increased according to the progression of dysplasia. Also, Ki-67 and p53 were predominantly expressed in the suprabasal layer of severe dysplasia and high risk of malignancy. Similar results were reported in studies evaluating the expression of these proteins in normal epithelium, OED and OSCC[23–25]. Studies have shown significant associations of Ki-67 and p53 in the dysplasia of high-risk of malignant transformation[16,26]. P53 is a tumor suppressor protein, which regulates the cell cycle, has also been involved in DNA repair and synthesis, cell proliferation, cell differentiation, programmed cell death, and in the maintenance of genomic stability[27]. Therefore, p53 when mutated may be a risk factor for malignant progression [27]. The percentage of Ki67 positive nuclei is often related to the clinical course of a disease, since it is present during all phases of the cell cycle except G0[26]. Caldeira et al.[16] suggests that morphological changes may be related to molecular changes in OEDs. Thus, our results reinforce the prognostic value of Ki-67 and p53 in OEDs.

It is known that the high subjectivity and variability in OED evaluation has been studied in the literature. Therefore, in our study, all cases were photographed by a single buccal pathologist experienced in magnifications of 200x and 400x and the microphotographs evaluated under the same software (Adobe photoshop, version 7.0) and in standardized contrasts. Thus, we ensured that all observers evaluated the same areas and under the same conditions to decrease inter- and intra-observer variability, increase reproducibility and improve diagnostic consensus.

In our study, intra-observer agreement analyzes were higher when compared to interobserver agreement in the individual assessment of morphological criteria (Table 4 and 5). The interobserver agreement scores are similar to those presented by Kujan et al. [6] and Nankivell et al. [28] with agreements ranging from negative disagreement to moderate. Previous studies[6,28] also presented greater consensus in

assessing architectural features. In contrast, Krishnan et al.[29] showed more consensus for the cytological criteria. Tilakaratne et al.[30] attributes the high interobserver variability to the limitation of statistical tests.

We also shown that the criteria such as increased nuclear size, atypical mitotic figures, increased nuclear cytoplasmic ratio, irregular epithelial stratification, increased number and size of nucleoli, keratin pearls within rete ridges, increased number of mitotic figures, abnormally superficial mitoses and premature keratinization in single cells were statistically associated with severe dysplasia and high risk of malignant transformation. Schepman et al. [32] presented similar results to our study evaluating malignant transformation in 166 patients with leukoplakia. Kujan et al.[11] evidence that loss polarity of basal cells, drop-shaped rete ridges, abnormally superficial mitoses, abnormal variation in nuclear shape, abnormal variation in cell size and shape, atypical mitotic figures and increased number and size of nucleoli are correlated to malignant transformation. Also, a recent study by our research group compared the OED criteria on actinic cheilitis and lip squamous carcinoma, the authors demonstrated similar results in exception of hyperchromatism which in this study was observed in 100% of cases [31]. Pilati et al [31] emphasize that these criteria should be carefully evaluated as they may indicate a worse prognosis since they are associated with high-risk dysplasia and carcinoma [31]. Our findings are consistent with this suggestion, since the criteria are poorly reproducible and associated with high-risk and severe dysplasia.

The results of our study reinforce the literature findings [6,12,28,33], pointing to higher scores of intra and interobserver agreement of the binary system compared to the WHO classification. Nankivell et al. [28] suggests that the high agreement presented by the binary system may be a result of the decrease in the number of categories, therefore, the fewer categories the greater the probability of consensus in the diagnosis. Although the WHO classification shows a lower concordance, our results indicate its similarity with binary system, since 61.5% and 100% of moderate and severe dysplasia, respectively, were classified as high risk of malignant transformation. Pilati et al. [31] showed a similarity between the two classification systems but suggested that the binary system may help pathologists to reach a consensus especially in critical cases because of their objectivity [31].

Previous studies pointed to sex and age as the main risk factors for malignant transformation of LOPMs to OSCC [5,34]. However, our results indicate that sex and age were not associated with OEDs. For

sex, these results can be explained by the similarity in the male/female ratio (1: 1.3). For age, perhaps because there are similar proportions and frequencies among the different groups of OEDs. Nayak et al.[35] studied LOPMs with a proven OED diagnosis and demonstrated that sex and age were not associated with OED [35].

Smoking is an important risk factor for the malignant transformation of LOPMs into OSCC [5]. According to Rivera [1], tobacco exposes the oral epithelium to oxygen and nitrogen free radicals, which can affect defense mechanisms and promote DNA mutations [1]. In the present study, it is important to emphasize that although without statistical difference, most OED patients were smokers and/or alcoholics. Perreira et al. [36] demonstrated a significant association between tobacco and OED. In the same way Nayak et al.[35], showed that tobacco habits increase the risk of epithelial dysplasia by 4.46-fold (95% CI = 1.47-12.75) [35].

The lesions at the border of the tongue have a high risk of malignant transformation. In our study, all lesions in the tongue were grouped due to the reduced number of cases. In the binary system these lesions are 4.32- fold more likely to present high risk dysplasia in relation to other intraoral sites. In WHO classification the chance decreases to 0.29-fold. This difference is due to the fact that all lesions graded according to the WHO classification in moderate (21.4%) and severe dysplasia (39.3%) were classified as high risk of malignant transformation in the binary system (60.7%). Joabe et al. did not a statistically significant association between the site of the lesion and the degree of dysplasia, however, points out that tongue lesions were more likely to present high-risk dysplasia. In summary, our results reinforce the consensus in the literature that lesions in tongue have a higher risk of malignant transformation in relation to other intraoral sites [34].

Non-homogeneous lesions were more likely to have high-risk (OR=9.25, 95% CI = 1,808-47,325, p = 0.008) and severe dysplasia (OR=10.50CI 95% = 1,893-58,242; p = 0.007) than homogeneous. In our study, the non-homogeneous lesions presented a lower prevalence (16.7%) compared to the homogeneous lesions (83.3%). Among the non-homogeneous lesions classified according to the WHO classification in moderate (18.2%) and severe dysplasia (63.6%), all were classified high-risk of malignant transformation (81.8%). Our study goes according to the consensus of the current literature indicating low prevalence and increased risk of malignant transformation for non-homogeneous lesions [34,37–39]. We reinforce the idea that histopathological evaluation and correlation of observations with

clinical parameters (clinical appearance of the lesion, smoking habits and lesion site) can provide a solid basis for clinical decision.

CONCLUSION

According to our results, the Ki-67 and p53 are useful molecular markers for OED prognosis and can be used as a complementary to the binary system. The binary system showed more consensus inter- and intra-observer compared to WHO classification. Furthermore, lesions with non-homogeneous clinical appearance and located in the tongue seemed better associated with high risk of malignant transformation of binary system.

Binary system appear be useful in differentiate those cases graded as moderate dysplasia. Therefore, we suggest the use of the binary system in the daily routine of histopathological diagnosis.

Reference

[1] Rivera C. Essentials of oral cancer. Int J Clin Exp Pathol 2015;8:11884–94. doi:10.1017/S0272263113000454.

[2] Hirshberg A, Berger R, Allon I, Kaplan I. Metastatic Tumors to the Jaws and Mouth. Head Neck Pathol 2014;8:463–74. doi:10.1007/s12105-014-0591-z.

[3] Villa A, Gohel A. Oral potentially malignant disorders in a large dental population. J Appl Oral Sci 2014;22:473–6. doi:10.1590/1678-775720140254.

[4] Reibel, Gale N, Hile J, Hunt JL, Lingen M, Muller S, et al. Oral potentially malignant disorders and oral dysplasia. WHO Classif. Head Neck Tumours, Lyon, 2017: 2017, p. 112–5.

[5] Warnakulasuriya S, Ariyawardana A. Malignant transformation of oral leukoplakia: a systematic review of observational studies. J Oral Pathol Med 2016;45:155–66. doi:10.1111/jop.12339.

[6] Kujan O, Khattab A, Oliver RJ, Roberts SA, Thakker N, Sloan P. Why oral histopathology suffers inter-observer variability on grading oral epithelial dysplasia : An attempt to understand the sources of variation. Oral Oncol 2007;43:224–31. doi:10.1016/j.oraloncology.2006.03.009.

[7] Mehanna H, Rattay T, Smith J, McConkey C. TREATMENT AND FOLLOW-UP OF ORAL DYSPLASIA — A SYSTEMATIC REVIEW AND META-ANALYSIS. Wiley Period Inc Head Neck 2009;36:1600–9. doi:10.1002/HED.

[8] Liu W, Bao ZX, Shi LJ, Tang GY, Zhou ZT. Malignant transformation of oral epithelial dysplasia: Clinicopathological risk factors and outcome analysis in a retrospective cohort of 138 cases. Histopathology 2011;59:733–40. doi:10.1111/j.1365-2559.2011.03938.x.

[9] Mehanna HM, Rattay T, Smith J, McConkey CC. TREATMENT AND FOLLOW-UP OF ORAL DYSPLASIA — A SYSTEMATIC REVIEW AND META-ANALYSIS. Head Neck 2009;36:1600–9. doi:10.1002/hed.21131.

[10] Le QT, Welch JJ, Vermorken JB, Rischin D, Mehanna H.

Formation of an international intergroup to coordinate clinical trials in head and neck cancers: HNCIG. Oral Oncol 2017;71:180–3. doi:10.1016/j.oraloncology.2017.05.022.

[11] Kujan O, Oliver RJ, Khattab A, Roberts SA, Thakker N, Sloan P. Evaluation of a new binary system of grading oral epithelial dysplasia for prediction of malignant transformation. Oral Oncol 2006;42:987–93. doi:10.1016/j.oraloncology.2005.12.014.

[12] Krishnan L, Karpagaselvi K, Kumarswamy J, Sudheendra U, Santosh K, Patil A. Inter- and intra-observer variability in three grading systems for oral epithelial dysplasia. J Oral Maxillofac Pathol 2016;20:261. doi:10.4103/0973-029X.185928.

[13] Žerdoner D. The Ljubljana classification - Its application to grading oral epithelial hyperplasia. J Cranio-Maxillofacial Surg 2003;31:75–9. doi:10.1016/S1010-5182(02)00186-5.

[14] Brothwell DJ, Lewis DW, Bradley G, Leong I, Jordan RCK, Mock D, et al. Observer agreement in the grading of oral epithelial dysplasia. Community Dent Oral Epidemiol 2003;31:300–5. doi:10.1034/j.1600-0528.2003.00013.x.

[15] Reibel J. Prognosis of Oral Pre-malignant Lesions: Significance of Clinical, Histopathological, and Molecular Biological Characteristics. Crit Rev Oral Biol Med 2003;14:47–62. doi:10.1177/154411130301400105.

[16] Caldeira PC, Abreu MHNG, Carmo MAV do. Binary system of grading oral epithelial dysplasia: Evidence of a bearing to the scores of an immunohistochemical study. J Oral Pathol Med 2012;41:452–3. doi:10.1111/j.1600-0714.2012.01128.x.

[17] Scholzen T, Gerdes J. The Ki-67 Protein : From the Known and 2000;322:311–22.

[18] Soares CP, Zuanon JAS, Teresa DB, Fregonezi PA, Neto CB, Oliveira MRB, et al. Quantitative cell-cycle protein expression in oral cancer assessed by computer-assisted system. Histol Histopathol 2006;21:721–8. doi:10.14670/HH-21.721.

[19] Zhang X, Kim KY, Zheng Z, Bazarsad S, Kim J. Nomogram for

risk prediction of malignant transformation in oral leukoplakia patients using combined biomarkers. Oral Oncol 2017;72:132–9. doi:10.1016/j.oraloncology.2017.07.015.

[20] Landis JR, Koch GG. The Measurement of Observer Agreement for Categorical Data. Biometrics 1977;33:159. doi:10.2307/2529310.

[21] Gonzalez-Moles MA, Ruiz-Avila I, Rodriguez-Archilla A, Martinez-Lara I. Suprabasal expression of Ki-67 antigen as a marker for the presence and severity of oral epithelial dysplasia. Head Neck 2000;22:658–61.

[22] Mithani SK, Mydlarz WK, Grumbine FL, Smith IM, Califano JA. Molecular genetics of premalignant oral lesions. Oral Dis 2007;13:126–33. doi:10.1111/j.1601-0825.2006.01349.x.

[23] Shailaja G, Kumar J, Baghirath P, Kumar U, Ashalata G, Krishna A. Estimation of malignant transformation rate in cases of oral epithelial dysplasia and lichen planus using immunohistochemical expression of Ki-67, P53, BCL-2, and BAX markers. Dent Res J (Isfahan) 2015;12:235–42.

[24] Birajdar S, Radhika M, Paremala K, Gadivan M, Sudhakara M, Soumya M. Expression of Ki-67 in normal oral epithelium, leukoplakic oral epithelium and oral squamous cell carcinoma. J Oral Maxillofac Pathol 2014;18:169. doi:10.4103/0973-029X.140729.

[25] Nasser W, Flechtenmacher C, Holzinger D, Hofele C, Bosch FX. Aberrant expression of p53, p16 INK4a and Ki-67 as basic biomarker for malignant progression of oral leukoplakias. J Oral Pathol Med 2011;40:629–35. doi:10.1111/j.1600-0714.2011.01026.x.

[26] Gadbail AR, Chaudhary MS, Sarode SC, Gawande M, Korde S, Tekade SA, et al. Ki67, CD105, and α-SMA expressions better relate the binary oral epithelial dysplasia grading system of World Health Organization. J Oral Pathol Med 2017;46:921–7. doi:10.1111/jop.12612.

[27] Vm R, Kamath A, Ra R. p53 immunoprofiling of potentially malignant oral disorders : A case series analysis 2012;49.

doi:10.4103/0019-509X.98913.

[28] Nankivell P, Williams H, Matthews P, Suortamo S, Snead D, McConkey C, et al. The binary oral dysplasia grading system: Validity testing and suggested improvement. Oral Surg Oral Med Oral Pathol Oral Radiol 2013;115:87–94. doi:10.1016/j.oooo.2012.10.015.

[29] Krishnan L, Karpagaselvi K, Kumarswamy J, Sudheendra U, Santosh K, Patil A. Inter- and intra-observer variability in three grading systems for oral epithelial dysplasia. J Oral Maxillofac Pathol 2016;20:261. doi:10.4103/0973-029X.185928.

[30] Tilakaratne WM, Sherriff M, Morgan PR, Odell EW. Grading oral epithelial dysplasia: Analysis of individual features. J Oral Pathol Med 2011;40:533–40. doi:10.1111/j.1600-0714.2011.01033.x.

[31] Pilati SFM, Bianco BC, Vieira DSC, Modolo F. Histopathologic features in actinic cheilitis by the comparison of grading dysplasia systems. Oral Dis 2017;23:219–24. doi:10.1111/odi.12597.

[32] Schepman KP, Van Der Meij EH, Smeele LE, Van Der Waal I. Malignant transformation of oral leukoplakia: A follow-up study of a hospital-based population of 166 patients with oral leukoplakia from The Netherlands. Oral Oncol 1998;34:270–5. doi:10.1016/S1368-8375(97)00097-3.

[33] Shubhasini AR, Praveen BN, Hegde U, Uma K, Shubha G, Keerthi G, et al. Inter- and Intra-Observer Variability in Diagnosis of Oral Dysplasia 2017;18:3251–4. doi:10.22034/APJCP.2017.18.12.3251.

[34] Speight PM, Khurram SA, Kujan O. Oral potentially malignant disorders: Risk of progression to malignancy. Oral Surg Oral Med Oral Pathol Oral Radiol 2018;125:612–27. doi:10.1016/j.oooo.2017.12.011.

[35] Nayak S, Chandra S, Mehrotra D, Kumar S, Agrawal SP, Kumar S, et al. Effect of tobacco, alcohol, and smoking habits in oral precancer with histological proven epithelial dysplasia. J Oral Biol Craniofacial Res 2012;2:159–62.

doi:10.1016/j.jobcr.2012.10.008.

[36] Pereira JDS, Carvalho MDV, Henriques ÁCG, De Queiroz Camara TH, Miguel MCDC, Freitas RDA. Epidemiology and correlation of the clinicopathological features in oral epithelial dysplasia: Analysis of 173 cases. Ann Diagn Pathol 2011;15:98–102. doi:10.1016/j.anndiagpath.2010.08.008.

[37] Mello FW, Miguel AFP, Dutra KL, Porporatti AL, Warnakulasuriya S, Guerra ENS, et al. Prevalence of oral potentially malignant disorders: a systematic review and meta-analysis. J Oral Pathol Med 2018:1–8. doi:10.1111/jop.12726.

[38] Yardimci G, Kutlubay Z, Engin B, Tuzun Y. Precancerous lesions of oral mucosa. World J Clin Cases 2014;2:866–72. doi:10.12998/wjcc.v2.i12.866.

[39] Mello FW, Melo G, Meurer MI, Rivero ERC. Intraoral Potentially Malignant Disorders in a Brazilian Oral Pathology Service: Epidemiological, Clinical, and Histopathological Findings. J Oncol 2018;2018. doi:10.1155/2018/2325808.

Figure 1Expression of Ki-67 and p53 in oral epithelial dysplasia.

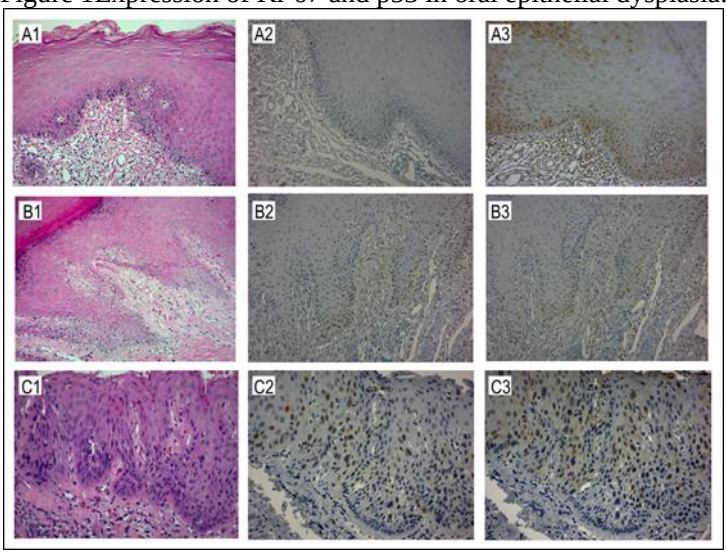

Figure1. Expression of Ki-67 and p53 in oral epithelial dysplasia. A1) Mild epithelial dysplasia / Low risk (H.E) (200x). A2) Nuclear expression of Ki-67 in the basal layer (200x). A3) Nuclear expression of p53 in the basal layer (200x). B1) Moderate epithelial dysplasia / Low risk (H.E) (200x). B2) Nuclear expression of Ki-67 in the basal layer (200x). B3) Nuclear expression of p53 in the basal layer (200x). C1) Severe epithelial dysplasia / High risk (H.E) (200x). C2) Nuclear expression of Ki-67 in the suprabasal layer (200x). C3) Nuclear expression of p53 in the suprabasal layer (200x).

Figure 2 Quantitative expression of Ki-67 and p53 in oral epithelial dysplasia.

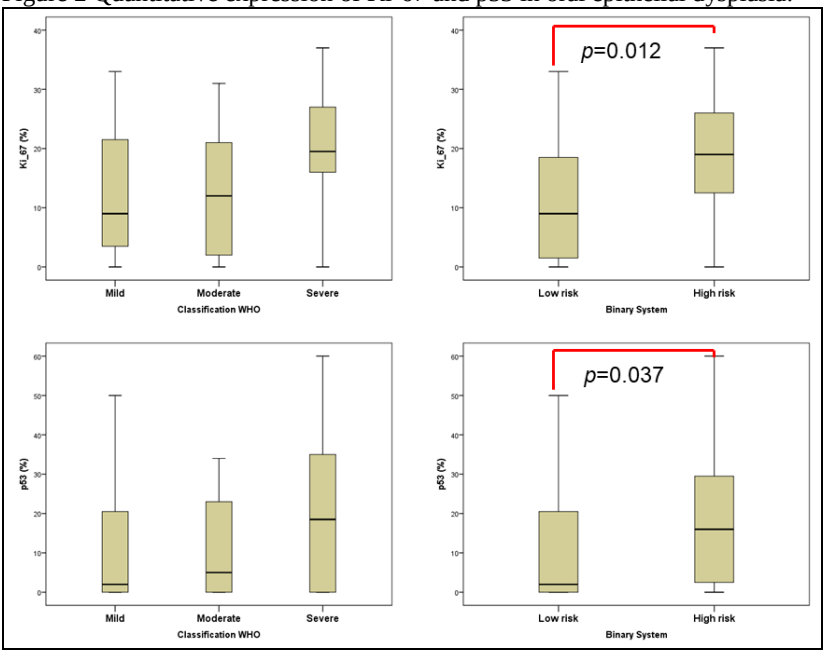

Figure 1.Comparison of quantitative expression of Ki-67 and p53 in the WHO classification (mild, moderate and severe) and in the binary system (low risk and high risk).

Table 1Cytological and architectural criteria used in the classification of oral epithelial dysplasia according to WHO (2017).

Cytological criteria	Architectural criteria
Abnormal variation in nuclear size	Irregular epithelial stratification
Abnormal variation in nuclear shape	Loss of polarity of basal cells
Abnormal variation in cell size	Drop-shaped rete ridges
Abnormal variation in cell shape	Keratin pearls within rete ridges
Increased nuclear cytoplasmic ratio	Increased number of mitotic figures
Increased nuclear size	Abnormally superficial mitoses
Atypical mitotic figures	Premature keratinization in single cells
Increased number and size of nucleoli	
Hyperchromatism	

Table 2 Demographic details and clinical- pathological data.

Characteristics	Frequency n (%)
Gender	
Male	28 (42.4)
Female	38 (57.6)
Age (mean ±SD)	56.8±12.9
Range	29-82
clinical appearance	
Homogeneous	55 (83.3)
Nonhomogeneous	11 (16.7)
Risk Factors	
Smoking	31 (46.9)
Alcohol	1 (1.5)
Smoking + Alcohol	20 (30.3)
No risk factor	8(12.1)
Missing cases	6 (9.0)
Intra-oral site	
Tongue	28 (42.4)
Floor of Mouth	5 (7.57)
Gingiva	3 (4.54)
Palate	8 (12.1)
Buccal mucosa	11 (16.6)
Retro molar	13 (19.7)
WHO OED grading	
Mild	35 (53.0)
Moderate	13 (19.7)
Severe/Carcinoma in situ	18 (27.3)
Binary grading	
Low- risk	39 (59.1)
High- risk	27 (40.9)

Table 3Reproducibility and agreement of each cytological criterion by intra- and-interobservers.

Cytological criteria	(A1vsA2)[2] kappa value 95% IC Agreement (%)	(B1vsB2)[1] kappa value 95% IC Agreement (%)	(A1vsB1)[3] kappa value 95% IC Agreement (%)	(A2vsB2)[4] kappa value 95% IC Agreement (%)
1. Abnormal variation in nuclear size	0.79 CI (0.39-1.00) 65 (97%)	0.78 CI (0.53-1.00) 61 (91%)	0.54 CI (0.24-.81) 64 (95.5%)	0.21 CI(-0.18-0.61) 65 (97%)
2. Abnormal variation in nuclear shape	0.71 CI (0.48-0.95) 57 (85.1%)	0.48 CI(-0.12-1.00) 65 (97%)	-0.05 CI(-0.11-0.01) 56 (83%)	-0.05 CI (-0.11-0.01) 57 (85.1%)
3. Abnormal variation in cell size	0.23 CI(-0.14-0.60) 66 (98.5%)	0.85 CI (0.55-1.00) 63 (94%)	0.14 CI(-0.19-0.48) 60 (89.6%)	0.38 CI(-0.15-0.92) 66 (98.5%)
4.Abnormal variation in cell shape	0.94 CI(0.83-1.00) 56 (83.6%)	0.73 CI (0.38-1.00) 62 (92.5%)	0.42 CI(0.09-0.75) 57 (85.1%)	0.44 CI(0.13-0.75) 56 (83.6%)
5. Increased nuclear cytoplasmic ratio	0.34 IC (0.10-0.57) 58 (86.6%)	0.63 IC (0.44-0.82) 41 (61.2%)	0.26 IC (0.02-0.51) 46 (68.7%)	0.25 IC(0.47-0.45) 58 (86.6%)
6. Increased nuclear size	0.35 CI (0.13-0.57) 52 (77.6%)	0.43 CI(0.10-0.75) 58 (86.6%)	0.16 CI(-0.02-0.35) 41 (61.2%)	0.19 CI(-0.07-0.46) 52 (77.6%)
7. Atypical mitotic figures	0.57 CI (0.24-0.90) 8 (11.9%)	0.74 CI (0.46-1.00) 6 (9%)	0.27 CI (-0.09-0.63) 5 (7.5%)	0.36 CI(0.01-0.71) 8 (11.9%)
8. Increased number and size of nucleoli	0.61 CI (0.42-0.80) 34 (50.7%)	0.40 CI (0.14-0.65) 13 (19.4%)	0.27 CI (0.07-0.48) 33(49.3%)	0.26 CI (0.080-0.44) 34 (50.7%)
9. Hyperchromatism	(100%)*	(100%)*	(100%)*	(100%)*

[1] intra-observer variability/Observer A
[2] intra-observer variability/Observer B
[3] inter-observer variability- first observation
[4] inter-observer variability- second observation (after 7 days)
*kappa value not calculated. There was agreement in all cases.

Table 4Reproducibility and agreement of each architecture criteria by intra-and-interobservers.

Architecture criteria	(A1vsA2)[2] kappa value 95% CI Agreement (%)	(B1vsB2)[1] kappa value 95% CI Agreement (%)	(A1vsB1)[3] kappa value 95% CI Agreement (%)	(A2vsB2)[4] kappa value 95% CI Agreement (%)
1.Irregular epithelial stratification	0.43 CI (0.19-0.66) 45 (67.2%)	1,0 CI (1.00-1.00) 66 (98.5%)	0.08 CI (-0.06-0.22) 49 (73.1%)	-0.03 CI (-0.08-0.02 45 (67.2%)
2. Loss of polarity of basal cells	0.36 CI (0.12-0.60) 9 (13.4%)	0.71 CI (0.53-0.80) 42(62.7%)	0.11 CI (-0.06-0.29) 20 (29.9%)	0.06 CI (-0.05-0.19) 9 (13.4%)
3. Drop-shaped rete ridges	0.64 CI (0.26-1.00) 63 (94%)	0.65 CI (0.20-1.00) 64 (95.5%)	-0.05 CI (-0.10-0.01) 62 (92.5%)	0.24 CI (-0.20-0.68) 63 (94%)
4. Keratin pearls within rete ridges	0.75 CI (0.38-1.00) 5 (7.5%)	0.56 CI (0.17-0.93) 4 (6%)	0.37 CI (-0.17-0.93 3 (4.5%)	0.47 CI (0.03-0.90) 5 (7.5%)
5. increased number of mitotic figures	0.66 CI (0.47-0.84 40 (59.7%)	0.77 CI (0.61-0.93) 25 (37.3%)	0.28 CI (0.08-0.48) 39 (58.2%)	0.45 CI (0.27-0.64) 40 (59.7%)
6. Abnormally superficial mitoses	0.72 CI (0.52-0.93) 15 (24.4%)	0.56 CI (0.19-0.94) 4 (6%)	0.22 CI (-0.06-0.50) 13 (19.4%)	0.24 CI (-0.01-0.50) 15 (22.4%)
7. Premature keratinization in single cells	0.49 CI (0.29-0.69) 38 (56.7%)	0.64 CI (0.45-0.82) 34 (50.7%)	0.31 CI (0.09-0.54) 31 (46.3%)	0.64 CI (0.45-0.82) 38 (56.7%)

[1] intra-observer variability/Observer A
[2] intra-observer variability/Observer B
[3] inter-observer variability- first observation
[4] inter-observer variability- second observation (after 7 days)

Table 5 Kappa value (95% confidence interval) and exact agreement for the WHO and binary systems.

Observer	**(B1vsB2)[1] kappa value 95% CI Agreement (%)**	**(A1vsA2)[2] kappa value 95% CI Agreement (%)**	**(B1vsA1)[3] kappa value 95% CI Agreement (%)**	**(B2vsA2)[4] kappa value 95% CI Agreement (%)**
WHO classification	0.93 [a] (0.86-0.99) 63 (94%)	0.77 [a] (0.66-.89) 54 (80.5%)	0.58 [a] (042-0.74) 45 (67.1%)	0.58 [a] (0.44-0.73) 43 (64.1%)
Binary system	1.0 (1.00-1.00) 67 (100%)	0.87 (0.76-0.99) 63 (94%)	0.61 (042-0.74) 54 (80.5%)	0.61 (0.42-0.80) 54 (80.5%)

[a]weighted kappa

[1] intra-observer variability/Observer B

[2] intra-observer variability/Observer A

[3] inter-observer variability- first observation

[4] inter-observer variability- second observation (after 7 days)

5 CONCLUSÃO

De acordo com os nossos resultados é possível concluir que:

1. O Sistema binário apresentou maior reprodutibilidade inter e intra observador quando comparado com a classificação segundo OMS.

2. Os critérios morfológicos de melhor reprodução foram hipercromatisco, disceratose, variação anormal de forma da célula, pérolas de ceratina, número aumentado de figuras mitoticas, variação anormal de forma do núcleo. Os critérios de pior reprodução foram variação anormal de forma do núcleo, estratificação epithelial irregualar e alteração de polaridade de células basais.

3. O Ki-67 e p53 foram expressos de acordo com o grau da displasia epitelial. No entanto, apenas o sistema binário foi estatisticamente associado com a expressão das proteínas ki-67 e p53.

4. Fatores de risco clínicos (lesões em língua e com aparência clínica não-homogênea) parecem estar associados com o sistema binário.

REFERÊNCIAS

ANGIERO, F. et al. Expression of P16, P53 and Ki-67 proteins in the progression of epithelial dysplasia of the oral cavity. **Anticancer Research**, v. 28, n. 5 A, p. 2535–2539, 2008.

ARDUINO, P. G. et al. Outcome of oral dysplasia: A retrospective hospital-based study of 207 patients with a long follow-up. **Journal of Oral Pathology and Medicine**, v. 38, n. 6, p. 540–544, 2009.

AWADALLAH, M. et al. Management update of potentially premalignant oral epithelial lesions. **Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology**, v. 125, n. 6, p. 628–636, 2018.

BARNES, L. et al. Pathology and Genetics of Head and Neck Tumours. **WHO Classification of Tumour**, n. 9, p. 163–175, 2005.

BIRAJDAR, S. et al. Expression of Ki-67 in normal oral epithelium, leukoplakic oral epithelium and oral squamous cell carcinoma. **Journal of Oral and Maxillofacial Pathology**, v. 18, n. 2, p. 169, 2014.

BROTHWELL, D. J. et al. Observer agreement in the grading of oral epithelial dysplasia. **Community Dentistry and Oral Epidemiology**, v. 31, n. 4, p. 300–305, 2003.

CALDEIRA, P. C.; ABREU, M. H. N. G.; CARMO, M. A. V. DO. Binary system of grading oral epithelial dysplasia: Evidence of a bearing to the scores of an immunohistochemical study. **Journal of Oral Pathology and Medicine**, v. 41, n. 6, p. 452–453, 2012.

CHANG, Y. C. et al. Invasive pattern grading score designed as an independent prognostic indicator in oral squamous cell carcinoma. **Histopathology**, v. 57, n. 2, p. 295–303, 2010.

CHUNG, C. et al. Oral precancerous disorders associated with areca quid chewing , smoking , and alcohol drinking in southern Taiwan. **J Oral Pathol Med**, v. 44, p. 460–466, 2005.

CRUZ et al. Suprabasal p53 immunoexpression is strongly associated with high grade dysplasia and risk for malignant transformation in potentially malignant oral lesions from Northern Ireland. **Journal of Clinical Pathology**, v. 55, n. 2, p. 98–104, 2002.

CRUZ, I. B. et al. P53 Expression Above the Basal Cell Layer in Oral Mucosa Is an Early Event of Malignant Transformation and Has Predictive Value for Developing Oral Squamous Cell Carcinoma. **J Pathol**, v. 184, n. 4, p. 360–368, 1998.

DOST, F. et al. A retrospective analysis of clinical features of oral malignant and potentially malignant disorders with and without oral epithelial dysplasia. **Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology**, v. 116, n. 6, p. 725–733, 2013.

FORMAN, D.; FERLAY, J. World Cancer Report 2014. In: BW, S.; WILD, C. (Eds.). . **International Agency for Research on Cancer, Lyon, France**. Lyon: [s.n.]. p. 16–69.

GADBAIL, A. R. et al. Ki67, CD105, and α-SMA expressions better relate the binary oral epithelial dysplasia grading system of World Health Organization. **Journal of Oral Pathology and Medicine**, v. 46, n. 10, p. 921–927, 2017.

GONZALEZ-MOLES, M. A. et al. Suprabasal expression of Ki-67 antigen as a marker for the presence and severity of oral epithelial dysplasia. **Head & neck**, v. 22, n. 7, p. 658–61, 2000.

HASHIBE, M. et al. Chewing Tobacco , Alcohol , and the Risk of Erythroplakia. **Cancer Epidemiology, Biomarkers & Prevention**, v. 9, n. July, p. 639–645, 2000.

HIRSHBERG, A. et al. Metastatic Tumors to the Jaws and Mouth. **Head and Neck Pathology**, v. 8, n. 4, p. 463–474, 2014.

HO, M. W. et al. The clinical determinants of malignant transformation in oral epithelial dysplasia. **Oral Oncology**, v. 48, n. 10, p. 969–976, 2012.

HUMAYUN, S.; PRASAD, VR. Expression of p53 protein and ki-67 antigen in oral premalignant lesions and oral squamous cell carcinomas: An immunohistochemical study. **National Journal of Maxillofacial Surgery**, v. 2, n. 1, p. 38, 2011.

IAMAROON, A. et al. Co-expression of p53 and Ki67 and lack of EBV expression in oral squamous cell carcinoma. **Journal of Oral Pathology and Medicine**, v. 33, n. 1, p. 30–36, 2004.

IATROPOULOS, M. J.; WILLIAMS, G. M. Proliferation markers. **Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie**, v. 48, n. 2–3, p. 175–181, 1996.

INCA. **Estimativa 2018-Incidência de câncer no Brasil**. Rio de Janeiro: [s.n.].

KANNAN, S. et al. Expression of p53 in leukoplakia and squamous cell carcinoma of the oral mucosa: correlation with expression of Ki67. **Clinical molecular pathology**, v. 49, n. 3, p. M170-5, 1996.

KRISHNAN, L. et al. Inter- and intra-observer variability in three grading systems for oral epithelial dysplasia. **Journal of Oral and Maxillofacial Pathology**, v. 20, n. 2, p. 261, 2016.

KUJAN, O. et al. Evaluation of a new binary system of grading oral epithelial dysplasia for prediction of malignant transformation. **Oral Oncology**, v. 42, n. 10, p. 987–993, 2006.

KUJAN, O. et al. Why oral histopathology suffers inter-observer variability on grading oral epithelial dysplasia : An attempt to understand the sources of variation. **Oral Oncology**, v. 43, n. 3, p. 224–231, 2007.

KUMAR et al. Prevalence and Risk Factors for Oral Potentially Malignant Disorders in Indian Population. **Advances in PreventiveMedicine independent**, v. 2015, 2015.

KUMAR, M. et al. Oral cancer : Etiology and risk factors : A review. **Journal of Cancer Research and Therapeutics**, v. 12, n. 2, p. 458–463, 2016.

LANDIS, J. R.; KOCH, G. G. The Measurement of Observer Agreement for Categorical Data. **Biometrics**, v. 33, n. 1, p. 159, 1977.

LANE, D. P. p53, guardian of the genome. **Nature**, v. 358, p. 15–16, 1992.

LE, Q. T. et al. Formation of an international intergroup to coordinate clinical trials in head and neck cancers: HNCIG. **Oral Oncology**, v. 71, p. 180–183, 2017.

LI, L. T. et al. Predic Ki67 is a promising molecular target in the diagnosis of cancer (Review). **Molecular Medicine Reports**, v. 11, n. 3, p. 1566–1572, 2015.

LIU, W. et al. Malignant transformation of oral leukoplakia: a retrospective cohort study of 218 Chinese patients. **BMC cancer**, v. 10, n. 1, p. 685, 2010.

LIU, W. et al. Malignant transformation of oral epithelial dysplasia: Clinicopathological risk factors and outcome analysis in a retrospective cohort of 138 cases. **Histopathology**, v. 59, n. 4, p. 733–740, 2011.

MAIA, H. C. DE M. et al. Potentially malignant oral lesions: Clinicopathological correlations. **Einstein (Sao Paulo)**, v. 14, n. 1, p. 35–40, 2016.

MEHANNA, H. et al. TREATMENT AND FOLLOW-UP OF ORAL DYSPLASIA — A SYSTEMATIC REVIEW AND META-ANALYSIS. **Wiley Periodicals, Inc. Head Neck**, v. 36, n. 10, p. 1600–1609, 2009a.

MEHANNA, H. M. et al. TREATMENT AND FOLLOW-UP OF ORAL DYSPLASIA — A SYSTEMATIC REVIEW AND META-ANALYSIS. **Head and Neck**, v. 36, n. 10, p. 1600–1609, 2009b.

MELLO, F. W. et al. Prevalence of oral potentially malignant disorders: a systematic review and meta-analysis. **Journal of Oral Pathology & Medicine**, n. April, p. 1–8, 2018a.

MELLO, F. W. et al. Intraoral Potentially Malignant Disorders in a Brazilian Oral Pathology Service: Epidemiological, Clinical, and Histopathological Findings. **Journal of Oncology**, v. 2018, 2018b.

MITHANI, S. K. et al. Molecular genetics of premalignant oral lesions. **Oral Diseases**, v. 13, n. 2, p. 126–133, 2007.

MOHAMMED, F.; FAIROZEKHAN, A. T. **Leukoplakia, Oral**. Treasure Island: [s.n.].

MONTERO, P.; G. PATEL, S. Cancer of the oral cavity. **Oxford Handbook of Oncology**, v. 24, n. 3, p. 491–508, 2015.

NAKAGAWA, K. et al. bc / -2 Expression in Epidermal Keratinocytic Diseases. **CANCER**, v. 74, n. 6, p. 1720–1724, 1994.

NANKIVELL, P. et al. The binary oral dysplasia grading system: Validity testing and suggested improvement. **Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology**, v. 115, n. 1, p. 87–94, 2013.

NAPIER et al. Potentially malignant oral lesions in Northern Ireland: size (extent) matters. **Oral diseases**, v. 9, n. 3, p. 129–37, 2003.

NAPIER, S. S.; SPEIGHT, P. M. Natural history of potentially malignant oral lesions and conditions: An overview of the literature. **Journal of Oral Pathology and Medicine**, v. 37, n. 1, p. 1–10, 2008.

NARAYAN, T.; SHILPASHREE, S. Meta-analysis on clinicopathologic risk factors of leukoplakias undergoing malignant transformation. **Journal of Oral and Maxillofacial Pathology**, v. 20, n. 3, p. 354, 2016.

NASSER, W. et al. Aberrant expression of p53, p16 INK4a and Ki-67 as basic biomarker for malignant progression of oral leukoplakias. **Journal of Oral Pathology and Medicine**, v. 40, n. 8, p. 629–635, 2011.

NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION (US). **Genes and Disease**. Bethesda (MD): NCBI, 1998.

NAYAK, S. et al. Effect of tobacco, alcohol, and smoking habits in oral precancer with histological proven epithelial dysplasia. **Journal of Oral Biology and Craniofacial Research**, v. 2, n. 3, p. 159–162, 2012.

PATTON, L. L. Oral cancer early detection: What will it take? **Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology**, v. 126, n. 1, p. 1–3, 2018.

PEREIRA, J. D. S. et al. Epidemiology and correlation of the clinicopathological features in oral epithelial dysplasia: Analysis of 173 cases. **Annals of Diagnostic Pathology**, v. 15, n. 2, p. 98–102, 2011.

PINDBORG, J. J.; REIBEL, J.; HOLMSTRUP, P. Subjectivity in evaluating oral epithelial dysplasia, carcinoma in situ and initial carcinoma. **Journal of Oral Pathology & Medicine**, v. 14, n. 9, p. 698–708, 1985.

RAM, H. et al. Oral Cancer: Risk Factors and Molecular Pathogenesis. **Journal of Maxillofacial and Oral Surgery**, v. 10, n. 2, p. 132–137, 2011.

REIBEL et al. Oral potentially malignant disorders and oral dysplasia. In: **WHO Classification of Head and Neck Tumours**. Lyon, 2017: IARC, 2017. p. 112–115.

REIBEL, J. Prognosis of Oral Pre-malignant Lesions: Significance of Clinical, Histopathological, and Molecular Biological Characteristics. **Crit Rev Oral Biol Med**, v. 14, n. 1, p. 47–62, 2003.

REICHART, P. A.; PHILIPSEN, H. P. Oral erythroplakia - A review. **Oral Oncology**, v. 41, n. 6, p. 551–561, 2005.

RIVERA, C. Essentials of oral cancer. **Int J Clin Exp Pathol**, v. 8, n. 9, p. 11884–11894, 2015.

RODRIGO, J. P. et al. Biomarkers predicting malignant progression of laryngeal epithelial precursor lesions: A systematic review. **European Archives of Oto-Rhino-Laryngology**, v. 269, n. 4, p. 1073–1083, 2012.

SABAPATHY, K.; LANE, D. P. Therapeutic targeting of p53: all mutants are equal, but some mutants are more equal than others. **Nature Reviews Clinical Oncology**, 2017.

SCHEPMAN, K. P. et al. Malignant transformation of oral leukoplakia: A follow-up study of a hospital-based population of 166 patients with oral leukoplakia from The Netherlands. **Oral Oncology**, v. 34, n. 4, p. 270–275, 1998.

SCHOLZEN, T.; GERDES, J. The Ki-67 Protein : From the Known and. v. 322, n. August 1999, p. 311–322, 2000.

SHAILAJA, G. et al. Estimation of malignant transformation rate in cases of oral epithelial dysplasia and lichen planus using immunohistochemical expression of Ki-67, P53, BCL-2, and BAX markers. **Dental research journal**, v. 12, n. 3, p. 235–42, 2015.

SHUBHASINI, A. R. et al. Inter- and Intra-Observer Variability in Diagnosis of Oral Dysplasia. v. 18, p. 3251–3254, 2017.

SILVA, I. C. O. et al. Leucoplasia : uma revisão de literatura. **RGO, Porto Alegre**, v. 55, n. 3, p. 287–289, 2007.

SILVEIRA, É. J. D. DA et al. Lesões orais com potencial de malignização: análise clínica e morfológica de 205 casos. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, v. 45, n. 3, p. 233–238, 2009.

SOARES, C. P. et al. Quantitative cell-cycle protein expression in oral cancer assessed by computer-assisted system. **Histology and Histopathology**, v. 21, n. 7–9, p. 721–728, 2006.

SPEIGHT, P. M.; KHURRAM, S. A.; KUJAN, O. Oral potentially malignant disorders: Risk of progression to malignancy. **Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology**, v. 125, n. 6, p. 612–627, 2018.

SUPOWIT, K. J. p53-INTEGRATING THE COMPLEXITY. **JOURNAL OF PATHOLOGY**, v. 13, n. 1, p. 182–196, 1984.

TILAKARATNE, W. M. et al. Grading oral epithelial dysplasia: Analysis of individual features. **Journal of Oral Pathology and Medicine**, v. 40, n. 7, p. 533–540, 2011.

VAN DER WAAL, I. Oral potentially malignant disorders: Is malignant transformation predictable and preventable? **Medicina Oral, Patologia Oral y Cirugia Bucal**, v. 19, n. 4, p. 1–5, 2014.

VILLA, A.; GOHEL, A. Oral potentially malignant disorders in a large dental population. **Journal of applied oral science : revista FOB**, v. 22, n. 6, p. 473–6, 2014.

VM, R.; KAMATH, A.; RA, R. p53 immunoprofiling of potentially malignant oral disorders : A case series analysis. v. 49, n. 1, 2012.

WANG, Y.-Y. et al. Malignant transformation in 5071 southern Taiwanese patients with potentially malignant oral mucosal disorders. **BMC Oral Health**, v. 14, n. 1, p. 99, 2014.

WARNAKULASURIYA, S. et al. Oral epithelial dysplasia classification systems: Predictive value, utility, weaknesses and scope for improvement. **Journal of Oral Pathology and Medicine**, v. 37, n. 3, p. 127–133, 2008.

WARNAKULASURIYA, S. et al. Factors predicting malignant transformation in oral potentially malignant disorders among patients accrued over a 10-year period in South East England. **Journal of Oral Pathology and Medicine**, v. 40, n. 9, p. 677–683, 2011.

WARNAKULASURIYA, S. Clinical features and presentation of oral potentially malignant disorders. **Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology**, v. 125, n. 6, p. 582–590, 2018.

WARNAKULASURIYA, S.; ARIYAWARDANA, A. Malignant transformation of oral leukoplakia: a systematic review of observational studies. **Journal of Oral Pathology & Medicine**, v. 45, n. 3, p. 155–166, 2016.

WEIJERS, M. et al. Malignancy grading is no better than conventional histopathological grading in small squamous cell carcinoma of tongue and floor of mouth: Retrospective study in 128 patients. **Journal of Oral Pathology and Medicine**, v. 38, n. 4, p. 343–347, 2009.

YARDIMCI, G. et al. Precancerous lesions of oral mucosa. **World journal of clinical cases**, v. 2, n. 12, p. 866–72, 2014.

ŽERDONER, D. The Ljubljana classification - Its application to grading oral epithelial hyperplasia. **Journal of Cranio-Maxillofacial Surgery**, v. 31, n. 2, p. 75–79, 2003.

ZHANG, X. et al. Nomogram for risk prediction of malignant transformation in oral leukoplakia patients using combined biomarkers. **Oral Oncology**, v. 72, p. 132–139, 2017.

APÊNDICE A – Metodologia expandida

DELINEAMENTO DO ESTUDO

O estudo foi do tipo observacional descritivo e retrospectivo. Os casos foram avaliados segundo a classificação da OMS e do sistema binário e submetidos à análise imunoistoquímica.

ASPECTOS ÉTICOS E LEGAIS

O presente estudo foi aprovado no Comitê de Ética em Pesquisa com Seres Humanos (CEPSH) da Universidade Federal de Santa Catarina (Plataforma Brasil- CAAE:42976715.3.0000.0121) sob parecer nº: 1.005.58730/03/2015).

COLETA DE DADOS

Realizou-se um levantamento nos arquivos do Laboratório de Patologia Bucal (LPB-UFSC) de casos de displasia epitelial intraoral diagnosticados clinicamente como leucoplasia, eritroplasia e eritroleucoplasia do ano 2007 a 2017. Em seguida todos os casos foram agrupados em lesões homogêneas e não homogêneas. Foram consideradas lesões homogêneas todas as lesões diagnosticadas como leucoplasias ou descritas clinicamente como lesões de coloração esbranquiçada, superfícies lisas, corrugadas ou com textura consistente. As lesões não homogêneas foram aquelas diagnosticadas clinicamente como eritroplasia, eritroleucoplasias ou leucoeritroplasias e aquelas descritas clinicamente como leucoplasias salpicadas.

Foram incluídos no estudo casos com fichas de biopsia adequadamente preenchidos, contendo as seguintes informações: localização da lesão, dados relativos a sexo, idade, fatores de risco (hábitos de fumar, álcool e fumantes/etilistas) (Quadro 1) e os seus respectivos blocos de parafina contendo material biológico suficiente para análise. Para casos com múltiplas biópsias, foi considerada a última realizada.

Foram excluídos do estudo casos que não preencheram os requisitos acima citados e sem consenso diagnóstico de displasia epitelial. Também, foram excluídos os casos que apresentaram infiltrado inflamatório em banda justa epitelial e que tenham tido como

diagnóstico clínico líquen plano ou reação liquenóide e de leucoplasia verrucosa proliferativa.

Todos os casos foram reexaminados e reclassificados segundo o sistema de classificação da OMS e pelo sistema binário. Por fim, foram incluídos no estudo 66 casos distribuídos em 35 displasias epiteliais orais leves (DEL), 13 displasias epiteliais orais moderadas (DEM) e 18 displasias epiteliais orais severas (DES). Para o sistema binário foram classificados em 39 DARM e 27 DBRM.

Para fins de correlação, foram levantados os seguintes dados clínicos (Quadro 1):

1.Quanto ao paciente: idade, gênero, hábitos de fumar e consumo de álcool.

2.Quanto à lesão: localização anatômica e características clínicas (tamanho).

Para tal, foi confeccionada uma base de dados digital para o armazenamento dessas informações para posterior análise descritiva e de correlação com o grau das displasias epiteliais e expressão das proteínas anti-Ki-67 e anti-p53 (Quadro 1):

AVALIAÇÃO DOS SISTEMAS DE CLASSIFICAÇÃO

Na primeira fase, para fins de padronização, todos os casos foram fotografados por um único avaliador em magnificações de 20x e 40x, de modo a garantir que todas as áreas displásicas serão avaliadas. Em seguida, as lâminas histopatológicas e as microfotografias foram codificadas aleatoriamente por meio de um sistema de rotulagem, cujos detalhes eram acessíveis apenas ao auxiliar não participante.

Na segunda fase, dois alunos do Programa de Pós-graduação em Odontologia – área de concentração em Diagnóstico Bucal, devidamente treinados avaliaram as microfotografias de forma independente, e em dois momentos diferentes num intervalo mínimo de 7 dias entre a 1ª e a 2ª avaliação de cada caso. Em casos de dúvidas na avaliação das microfotografias, as lâminas histopatológicas coradas com hematoxilina e eosina foram avaliadas com o uso do microscópio óptico de luz.

Todos os casos em desacordo na classificação final, foram reavaliados por terceiro avaliador (Professora de Patologia Bucal da UFSC) para acordo no diagnóstico final. Todas as avaliações foram realizadas de forma independente sem o conhecimento prévio do diagnóstico clínico, histopatológico e dos dados clínicos.

Os casos foram classificados com o uso do sistema da OMS em DEL (presença de alterações celulares e arquiteturais restritas ao terço

basal do epitélio) DEM (quando as alterações se estendem até o terço médio do epitélio afetado) e DES (quando as alterações ultrapassam o terço médio e estendem-se pelo terço superior). Os casos também foram classificados com o uso do sistema binário em DEA (presença de no mínimo, quatro critérios arquiteturais e cinco critérios citológicos no epitélio) e DEB (menos de 4 arquiteturais e 5 critérios citológicos no epitélio). Todos os critérios arquiteturais e citológicos (Tabela 1) foram avaliados de forma independente, observando a sua reprodutibilidade inter e intra-observador (Tabela 1).

Para fins de comparação e correlação com as demais variáveis em estudo, foi confeccionada uma ficha padronizada e categorizada para ambos os sistemas de classificação.

PROCEDIMENTOS LABORATORIAIS

Foram realizados cortes teciduais de 3µm de espessura, montados em lâminas preparadas com solução de ATPS (3-aminopropyltriethoxysilene). Os cortes foram inicialmente fixados na lâmina mantendo-os em estufa a 65ºC, durante 3 horas. Após, as lâminas foram submetidas a dois banhos de xilol em temperatura ambiente (no primeiro banho foram imersas overnight e, no segundo, durante 20 minutos). Posteriormente, foram realizados três banhos em soluções decrescentes de álcool etílico (absoluto, 95% e 85%), seguidos de dois banhos em água destilada, todos de 5 minutos cada. As lâminas foram imersas em solução para bloqueio da peroxidase endógena (80ml de álcool metílico + 20ml de Peróxido de Hidrogênio -H2O2), com dois banhos de 20 minutos cada.

A reativação antigênica foi realizada mantendo as lâminas em solução de tampão citrato 0,01M, pH 6,0, em banho-maria com a temperatura constante de 96ºC, durante 40 minutos. Os sítios não específicos foram bloqueados com leite desnatado (5%) em solução de tampão fosfato-salino (PBS) por 40 min. A incubação com os anticorpos primários anti-p53 e anti-Ki67 (Tabela 3) foi realizada em câmara úmida mantida sob refrigeração (4 a 8°C), durante 18 horas.

Para amplificação da reação foi utilizado o kit de marcação envision- Dako, que consta de soro secundário policlonal e do soro terciário streptavidina-biotina com peroxidase conjugada. A revelação da reação foi realizada através de solução cromógena, contendo diaminobenzidina (DAB) em tampão Tris-HCl 0,05M, pH 7,4, e adicionando peróxido de hidrogênio a 0,3%. Após a revelação, foi realizada a contra coloração dos cortes com hematoxilina de Harris (2,5

minutos), seguido de desidratação em cadeias de concentração crescentes de etanol (etanol 50% a etanol absoluto), diafanização em xilol e montagem com Entellan (Merck, Alemanha). Após montadas, as lâminas foram mantidas em estufa (40ºC) por, no máximo, 2 horas, antes de serem examinadas ao microscópio de luz.

Tabela 2 Descrição das características dos anticorpos utilizados nas reações de imunoistoquímica.

Anti-corpo	Fabricante/ Marca	Origem	Diluído	Padrão de Coloração	Controle Positivo
p53	Sigma Aldrich	Monoclonal de camundongo	1:400	Nuclear	Carcinoma epidermoide
Ki-67	Spring Bioscience	Monoclonal de coelho	1:400	Nuclear	Carcinoma epidermoide

ANÁLISE IMUNO-HISTOQUÍMICA

A marcação do Ki-67 e p53 é nuclear. Portanto, foram consideradas positivas todas as células epiteliais com marcação castanha/marrom no núcleo, independentemente da intensidade da coloração, excluindo as células inflamatórias.

Para avaliação das reações imunoistoquímicas, foi utilizado o software NIH ImageJ 1.45q (National Institutes of Health, Maryland, EUA) a partir de imagens capturadas com câmera fotográfica (Cannon, A620, San Jose, CA, USA) acoplada a microscópio de luz (Axiostar Plus, Carl Zeiss, Oberkochen, Alemanha), com magnitude de 400X.

O índice de Ki-67 e p53 foi determinado pela porcentagem de células positivas em 10 campos de 400x (Iamaroon *et al.*, 2004). Para analisar a expressão do Ki-67 e p53, foram avaliados os seguintes parâmetros:

1. A porcentagem de células positivas.
2. Padrão de coloração das camadas de células epiteliais em cada caso e grupo, em relação a distribuição/local de marcação na camada basal e suprabasal.

As informações obtidas nas análises foram armazenadas em uma planilha do software Microsoft Excel® (Microsoft Office Corporation, EUA). Posteriormente, transferidos para o software IBM SPSS statistics 21 (IBM SPSS Statistics 21 de 2014, EUA) para análise estatística.

ANÁLISE ESTATÍSTICA

A variabilidade intra e inter-observador de cada critério morfológico (Tabela 1) e do sistema binário foi avaliada com o uso do teste Kappa não ponderada. Para o sistema da OMS foi calculado o kappa ponderada. Os kappas não ponderados fornecem uma associação baseada apenas na concordância entre avaliadores. O Kappa ponderado é utilizado para calcular a reprodutibilidade quando as variáveis são ordinais e os resultados expressos por mais de duas categorias, também, confere maior importância à discordância, atribuindo-lhe maior peso no cálculo da reprodutibilidade. Os valores do Kappa foram atribuídos de acordo com LANDIS; KOCH (1977) (Tabela 3).

Para realizar a comparação entre os grupos de DEL, DEM e DES, a partir dos resultados da análise quantitativa dos anticorpos Ki-67 e p53, para cada anticorpo estudado, foi realizado o teste estatístico de Kruskal-Wallis, teste não paramétrico para comparar 3 ou mais amostras independentes. Para comparar os resultados entre as DARM e DBRM, foi aplicado o teste estatístico de Mann Whitney, teste não paramétrico para comparar 2 amostras independentes.

Tabela 3Valores do Kappa de acordo com Landis e Koch (1977).

Valor de Kappa	Interpretação
<0	Sem acordo
0-0.19	Acordo pobre
0.20-0.39	Acordo justo
0.40-0.59	Acordo moderado
0.60-0.79	Acordo substancial
0.80-1.00	Acordo quase perfeito

Teste qui-quadrado de duas vias e o teste exato de Fisher foram usados para analisar as associações entre as variáveis de interesse (classificação da OMS e sistema binário).

Uma análise de regressão logística binária e multinominal foi realizada para avaliar a associação dos fatores de risco com os sistemas de classificação das DEO. No entanto, as variáveis como diagnóstico clínico, localização da lesão, fatores de risco clínicos (hábitos de fumar e álcool/fumo), idade e sexo foram adicionados em ambos os modelos de forma isolada (análise bruta) e combinada (análise ajustada). Na análise multivariada (analise ajustada) foram adicionadas apenas variáveis com p> 0,20.

APÊNDICE B- Características clínicas e fatores de risco dos casos estudados

Tabela 4Resumo das caracteristicas clinicas e e fatores de risco dos casos estudados.

Características clínicas e fatores de risco	Homogênea 55 (83,3%)	Não homogênea 11 (10,6%)	Total 66 (100%)
Gênero			
Masculino	23 (82,1%)	5 (17,9%)	28 (100%)
Feminino	32 (84,2%)	6 (15,8%)	38 (100%)
Idade (média ±D.P)	56,5±13,7	58,7±9,6	56,8±12,9
Intervalo	29-82	45-74	29-82
Tamanho (média±D.P)	22,3mm±15,9	26,2mm±5,8	21,7mm±14,7
Intervalo	5mm-120mm	7mm-27mm	5mm-120mm
Fatores de Risco			
Tabaco	30 (96,8%)	1 (3,2%)	31 (100%)
Álcool	1 (100%)	0 (0,0%)	1 (100%)
Tabaco + Álcool	11 (55,0%)	9 (45,0%)	20 (100%)
Sem fator de risco	8 (100%)	0 (0,0%)	8 (100%)
Dados perdidos	4(66,7%)	2 (33,3%)	6 (100%)
Localização intra-oral			
Língua	22(78,6%%)	6 (21,4%)	28 (100%)
Assoalho de boca	5 (100%)	0 (0,0%)	5 (100%)
Gengiva	3 (100%)	0 (100%)	3 (100%)
Palato	7 (87,5%)	1 (12,5%)	8 (100%)
Mucosa bucal	8 (72,7%)	3 (27,3%)	11(100%)
Retromolar	11 (78,6%)	2 (21,4%)	13 (100%)
Classificação da OMS			
Leve	33 (94,3%)	2 (5,7%)	35 (100%)
Moderado	11 (84,6%)	2 (15,4%)	13 (100%
Severa/Carcinoma *in situ*	11 (61,1%)	7 (38,9%)	18 (100%)
Sistema binário			
Alto risco	18 (66,7%)	9(33,3%)	27 (100%)
Baixo Risco	37(94,9%)	2(5,1%)	39 (100%)
Expressão de Ki-67	16,5±11,83	10,1±8,7	----------
Expressão de P53	14,8±17,4	12,8±16	----------

Tabela 5 Resultados da regressão logística binária bruta e ajustada. Associação dos fatores de risco com o sistema binário de alto risco.

Variáveis	Sistema binário				
	Casos n (%)	**Não ajustado**		**Ajustado**	
		OR (95% C.I)	p-valor	OR (95% C.I)	p-valor
Sexo					
Masculino	28 (42.4)	1	0.462		
Feminino	38 (57.6)	1.457 (0.534-3.973)			
Idade					
<45	14 (21.2)	1	0.295		
>46	52(78.8)	1.983 (0.550-7.147)			
Fatores de risco					
Tabaco					
Não	8 (12.1)	1			
Sim	51 (77.2)	1.030 (0.229-4.638)	0.969		
Tabaco + Álcool					
Não	45 (68.2)	1			
Sim	21(31.8)	2.667 (0.921-7.724)	0.071	0.681 (0.138-3.352)	0.637
Localização intra-oral					
Retromolar					
Não	53 (80.4)	1			
Sim	13 (19.6)	0.504 (0.140-1.818)	0.295		
Língua					
Não	38(57.6)	1			
Sim	28 (42.4)	4.327 (1.519-12.331)	**0.006[a]**	3.621 (1.119-11.718)	**0.032[a]**
Assoalho de boca					
Não	61(92.4)	1			
Sim	5 (7.6)	0.337 (0.035-3.191)	0.343		
Palato					
Não		1			
Sim	8 (12.1)	0.850 (0.185-3.901)	0.834		
Mucosa bucal					
Não	58 (87.9)	1			
Sim	11 (16.7)	0.484 (0.116-2.024)	0.320		
Aparência clínica					
Homogênea					
Não	11(16.7%)	1			
Sim	55(83.3%)	0.108 (0.021-0.553)	**0.008[a]**	0.034 (0.003-0.451)	**0.010[a]**
Não homogênea					
Não	55(83.3%)	1			
Sim	11(16.7%)	9.250 (1.808-47.325)	**0.008[a]**	6.460(1.119-37.296)	**0.037[a]**

Tabela 6Associação dos fatores de risco com a classificação segundo OMS. Resultados da regressão logística multinominal bruta, considerando a razão de chance para displasia leve.

		DEM* x DEL** Odds Ratio (OR) IC 95%	p-valor	DES*** x DEL Odds Ratio (OR) IC 95%	p-valor
Sexo	Masc.	1	0.121	1	0.776
	Fem.	0.318 (0.074-1.355)		0.847 (0.270-2.653)	
Idade	>45	1	0.200	1	0.780
	<45	0.241(0.027-2.122)		0.825 (0.215-3.168)	
Fatores de risco					
Tabaco	Não	1	0.422	1	0.937
	Sim	0.403 (0.044-3.713)		1.074 (0.184-6.283)	
Tabaco + Álcool	Não	1	0.851	1	0.082
	Sim	1.154(0.258-5.153)		0.346 (0.105-1.144)	
Localiz. intra-oral					
Retromolar	Não	1	0.454	1	0.459
	Sim	1.904 (0.353-0.283)		1.731 (0.405-7.401)	
Língua	Não	1	0.345	1	0.042[a]
	Sim	0.535 (0.145-1.968)		0.292 (0.089-0.955)	
Palato	Não	1	0.035[a]	1	0.489
	Sim	0.136 (0.021-0.869)		0.485 (0.062-3.762)	
Mucosa bucal	Não	1	0.474	1	0.370
	Sim	0.353 (0.20-6.095)		2.370 (0.447-12.271)	
Aparência clínica					
Homogênea	Não	1	0.474	1	0.007[a]
	Sim	0.353 (0.20-6.095)		0.095 (0.017-0.528)	
Não-homogênea	Não	1	0.299	1	0.007[a]
	Sim	3.000(0.377-23.902)		10.500(1.893-58.242)	

Tabela 7Associação dos fatores de risco com a classificação segundo OMS. Resultados da regressão logística multinominal ajustada, considerando a razão de chance para displasia leve.

		DEM* x DEL** Odds Ratio (OR) IC 95%	p-valor	DES*** x DEL Odds Ratio (OR) IC 95%	p-valor
Sexo	Masc.	1	0.175	1	0.852
	Fem.	0.303(0.054-1.704)		0.868(0.195-3.868)	
Fatores de risco					
Tabaco + Álcool	Não	1	0.751	1	0.792
	Sim	1.388(0.183-10.512)		0.795(0.145-4.363)	
Localiz. intra-oral					
Língua	Não	1	0.123	1	**0.038[a]**
	Sim	0.271 (0.052-1.422)		0.227 (0.056-0.923)	
Palato	Não	1	**0.009[a]**	1	0.213
	Sim	0.059 (0.007-0.499)		0.224 (0.021-2.357)	
Aparência clínica					
Homogênea	Não	1	0.761	1	0.670
	Sim	1.960(0.026-149.937)		0.483 (0.017-13.664)	
Não-homogênea	Não	1	0.324	1	0.182
	Sim	6.205(0.165-233.742)		7.095 (0.400-125.975)	

Quadro 1Apresenta características clínicas e média de porcentagem do Ki-67 e p53 para cada caso estudado.

Sexo	Diagnóstico Clínico	Idade	Fatores de Risco	Tamanho	Localização Anatômica	Classificação OMS	Sistema Binário	Ki-67	P53
Feminino	Homogênea	50	Tabagista	9	Língua	DEL	DBRM	15	21
Feminino	Homogênea	46	Tabagista	11	Mucosa jugal	DEL	DBRM	26	28
Masculino	Homogênea	54	Tabagista	5	Assoalho de boca	DEL	DBRM	10	0
Feminino	Homogênea	33	Tabagista	20	Gengiva inserida	DEL	DBRM	18	2
Masculino	Homogênea	41	Tabagista	120	Mucosa jugal	DEL	DBRM	24	21
Masculino	Homogênea	47	Tabagista	24	retromolar	DEL	DBRM	8	3
Masculino	Homogênea	70	Tabagista	20	retromolar	DEL	DBRM	11	25
Feminino	Homogênea	60	Sem hábitos	21	Língua	DEL	DBRM	31	0
Masculino	Homogênea	65	Tabagista	21	Palato	DEL	DBRM	30	50
Masculino	Homogênea	70	Tabagista	33	retromolar	DEL	DBRM	19	33
Feminino	Homogênea	76	Tabagista	18	Língua	DEL	DBRM	29	47
Feminino	Homogênea	N. I	Tabagista	20	Mucosa jugal	DEL	DBRM	28	41
Feminino	Homogênea	74	Tabagista	25	Língua	DEL	DBRM	8	0
Masculino	Homogênea	46	Tabagista/Etlista	29	retromolar	DEL	DBRM	0	0
Masculino	Homogênea	80	Tabagista/Etlista	20	Mucosa jugal	DEL	DBRM	3	0
Masculino	Homogênea	50	Tabagista/Etlista	17	retromolar	DEL	DBRM	25	0

Feminino	Homogênea	35	Tabagista	16	Gengiva inserida	DEL	DBRM	1	0
Masculino	Homogênea	80	Tabagista	11	retromolar	DEL	DBRM	9	0
Masculino	Homogênea	51	Tabagista/Etlista	10	Palato	DEL	DBRM	1	1
Masculino	Homogênea	29	Tabagista	16	Assoalho de boca	DEL	DBRM	5	17
Masculino	Homogênea	45	Sem hábitos	10	retromolar	DEL	DBRM	9	0
Feminino	Homogênea	38	Tabagista	12	retromolar	DEL	DBRM	14	16
Feminino	Homogênea	79	Sem hábitos	34	Língua	DEL	DBRM	33	0
Feminino	Homogênea	60	Tabagista	40	Mucosa jugal	DEL	DBRM	26	27
Feminino	Não homogênea	62	Tabagista/Etlista	11	Língua	DEL	DBRM	2	2
Feminino	Homogênea	44	Sem hábitos	17	Mucosa jugal	DEL	DBRM	4	19
Masculino	Homogênea	58	Tabagista/Etlista	16	Língua	DEL	DBRM	10	0
Masculino	Homogênea	57	Tabagista/Etlista	19	Língua	DEL	DARM	5	16
Feminino	Homogênea	56	Sem hábitos	27	Mucosa jugal	DEL	DBRM	0	20
Masculino	Não homogênea	49	Tabagista/Etlista	7	retromolar	DEL	DBRM	0	0
Feminino	Homogênea	78	Sem hábitos	19	Língua	DEL	DBRM	0	0
Masculino	Homogênea	49	Tabagista	22	Mucosa jugal	DEL	DBRM	8	15
Feminino	Homogênea	N.I	Tabagista/Etlista	12	Assoalho de boca	DEL	DBRM	0	0
Feminino	Homogênea	60	Tabagista	13	Assoalho de boca	DEL	DBRM	11	5

Feminino	Homogênea	55	Tabagista	17	Língua	DEL	DBRM	8	0
Feminino	Homogênea	54	Tabagista	48	retromolar	DEM	DBRM	17	23
Feminino	Homogênea	51	Tabagista	5	Palato	DEM	DBRM	0	0
Feminino	Homogênea	62	Tabagista	30	Mucosa jugal	DEM	DARM	7	4
Masculino	Homogênea	50	Tabagista/Etlista	21	Língua	DEM	DBRM	0	0
Feminino	Homogênea	47	Tabagista	20	Assoalho de boca	DEM	DARM	17	19
Feminino	Homogênea	53	Tabagista	29	retromolar	DEM	DARM	31	25
Feminino	Não homogênea	56	Tabagista/Etlista	27	Língua	DEM	DARM	21	34
Masculino	Homogênea	64	Tabagista	21	Palato	DEM	DBRM	1	0
Feminino	Homogênea	43	Tabagista	15	Palato	DEM	DBRM	12	11
Feminino	Homogênea	77	Sem hábitos	13	Língua	DEM	DARM	21	29
Feminino	Não homogênea	59	Tabagista	20	Mucosa jugal	DEM	DARM	2	5
Masculino	Homogênea	54	Tabagista/Etlista	16	Língua	DEM	DARM	10	3
Feminino	Homogênea	79	Tabagista	20	Língua	DEM	DARM	31	0
Masculino	Homogênea	34	Etilista	20	retromolar	DES	DARM	29	5
Masculino	Não homogênea	60	Tabagista/Etlista	21	Mucosa jugal	DES	DARM	2	0
Feminino	Não homogênea	45	Tabagista/Etlista	25	Língua	DES	DARM	16	35
Feminino	Homogênea	48	Perdido	18	Língua	DES	DARM	19	21

Feminino	Homogênea	67	Perdido	31	Língua	DES	DARM	21	28
Masculino	Não homogênea	52	Tabagista/Etlista	15	Mucosa jugal	DES	DARM	15	0
Feminino	Não homogênea	74	Perdido	20	Língua	DES	DARM	16	35
Feminino	Não homogênea	65	Perdido	20	Retromolar	DES	DARM	0	0
Masculino	Homogênea	50	Perdido	18	Língua	DES	DARM	17	4
Feminino	Homogênea	N.I	Tabagista/Etlista	12	Assoalho de boca	DES	DARM	27	60
Masculino	Não homogênea	63	Tabagista/Etlista	23	Retromolar	DES	DARM	16	0
Masculino	Homogênea	50	Tabagista/Etlista	42	Língua	DES	DARM	37	40
Feminino	Homogênea	66	Perdido	26	Língua	DES	DARM	3	2
Feminino	Homogênea	60	Tabagista/Etlista	22	Palato	DES	DARM	31	25
Feminino	Homogênea	82	Sem hábitos	21	Língua	DES	DARM	25	16
Masculino	Não homogênea	60	Tabagista/Etlista	20	Palato	DES	DARM	21	30
Feminino	Homogênea	67	Tabagista	27	Língua	DES	DARM	20	0
Masculino	Homogênea	42	Tabagista	29	Língua	DES	DARM	35	45

ANEXO A- Parecer do Comitê de Ética em Pesquisa com Seres Humanos da UFSC

UNIVERSIDADE FEDERAL DE SANTA CATARINA - UFSC

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: O papel do estroma no desenvolvimento e progressão do câncer de boca
Pesquisador: Elena Riet Correa Rivero
Área Temática:
Versão: 1
CAAE: 42976715.3.0000.0121
Instituição Proponente: Departamento de Patologia
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.005.587
Data da Relatoria: 30/03/2015

Apresentação do Projeto:
Trata-se de projeto vinculado à linha de pesquisa "Etiologia, Diagnóstico, Prevenção e Terapias aplicadas à Odontologia", do Programa de Pós-graduação em Odontologia da UFSC. A professora coordenadora faz parte do grupo de Pesquisa em Diagnóstico Bucal da UFSC. O projeto desdobrar-se-á em uma tese de doutorado e um Trabalho de Conclusão de Curso. Como amostra positiva de neoplasia invasiva serão incluídos casos de carcinoma epidermóide de boca (CEB) e como amostra de tecido não neoplásico serão incluídos casos de HFI (hiperplasia fibrosa inflamatória). A seleção dos casos será feita com base no diagnóstico histopatológico e na análise das lâminas coradas em H&E. Com base na casuística desse Serviço de Diagnóstico espera-se no final ao menos 25 casos de DEBM; 25 casos de DEBM, 20 casos de carcinoma epidermóides de boca e 20 casos de HFI.

Objetivo da Pesquisa:
Objetivo Primário:
- O objetivo principal deste projeto é contribuir com o entendimento sobre o processo de invasão do CEB (carcinoma epidermóide de boca), por meio do estudo das interações parênquima/estroma nos mecanismos de crescimento e invasão tumoral.

Objetivo Secundário:
1- Promover um levantamento dos laudos histopatológicos de lesões diagnosticadas como displasias epiteliais, CEB e hiperplasia fibrosa inflamatória (HFI), presentes nos arquivos do

Endereço: Universidade Federal de Santa Catarina, Prédio Reitoria II (Edifício Santa Clara), R: Desembargador Vitor Lima,
Bairro: Trindade **CEP:** 88.040-400
UF: SC **Município:** FLORIANOPOLIS
Telefone: (48)3721-6094 **E-mail:** cep.propesq@contato.ufsc.br

Laboratório de Patologia Bucal (LPB) da Universidade Federal de Santa Catarina (UFSC);
2- Proceder a avaliação histológica dos casos selecionados e Classificar as displasias epiteliais segundo o sistema binário, em displasias de alto risco de
malignização (DEAM) e baixo risco de malignização (DEBM);
3- Investigar a presença de fibroblastos senescentes, por meio de marcadores de senescência celular (p16 e beta galactosidase), assim como por meio de marcadores de FAC (podoplanina), na lâmina própria de DEBM, DEAM e HFI, assim como no estroma de CEB.
4- Investigar a expressão de caveolina-1, osteopontina e MMP-2 na lâmina própria de DEBM, DEAM e HFI, e no CEB.
5- Estabelecer o índice de proliferação epitelial, por meio da marcação do antígeno Ki-67, em DEBM, DEAM, HFI e CEB;
6- Comparar a expressão das proteínas em estudo nos casos de DEBM, DEAM, HFI e CEB;
7- Comparar a expressão das proteínas em estudo nos casos de displasias epiteliais que evoluíram para carcinoma epidermóide;
8- Fazer a correlação das proteínas em estudo nos casos de DEBM, DEAM, HFI e
CEB.
9- Correlacionar os achados deste estudo com os já existentes na literatura.

Avaliação dos Riscos e Benefícios:

Em relação aos riscos da pesquisa, os pesquisadores esclarecem que "Durante a pesquisa será apenas utilizado o material resultante de biópsia da lesão, previamente realizada, o qual encontra-se armazenado nos arquivos do LPB, sem causar qualquer tipo de desconforto aos pacientes. Como haverá acesso aos dados presentes nas fichas de biopsia e laudos histopatológicos, há um risco de perda de sigilo dessas informações, mas os pesquisadores garantem que tomarão todos os cuidados para evitar
que isso ocorra".

No que se refere aos benefícios do estudo, observa-se que "envolvem a produção de conhecimento científico podendo servir de base para outros estudos, e possivelmente tentar ajudar os próximos pacientes que tenham a mesma doença no futuro, facilitando o seu diagnóstico".

Comentários e Considerações sobre a Pesquisa:

Sem comentários adicionais.

UNIVERSIDADE FEDERAL DE SANTA CATARINA - UFSC

Considerações sobre os Termos de apresentação obrigatória:

Todos os documentos necessários ao processo estão disponíveis na Plataforma Brasil e de acordo com a legislação vigente: folha de rosto; projeto de pesquisa; informações detalhadas sobre o projeto, incluindo cronograma e orçamento; e termo de consentimento livre e esclarecido (TCLE) a ser apresentado aos participantes da pesquisa.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

De acordo com o exposto nesse parecer, o projeto de pesquisa "O papel do estroma no desenvolvimento e progressão do câncer de boca" deve ser considerado APROVADO.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

FLORIANOPOLIS, 30 de Março de 2015

Assinado por:
Washington Portela de Souza
(Coordenador)

Endereço: Universidade Federal de Santa Catarina, Prédio Reitoria II (Edifício Santa Clara), R: Desembargador Vitor Lima,
Bairro: Trindade **CEP:** 88.040-400
UF: SC **Município:** FLORIANOPOLIS
Telefone: (48)3721-6094 **E-mail:** cep.propesq@contato.ufsc.br

ANEXO B- Normas da revista Oral Oncology

ORAL ONCOLOGY
A Journal Related to Head & Neck Oncology

ELSEVIER

AUTHOR INFORMATION PACK

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ISSN: 1368-8375

DESCRIPTION

Oral Oncology is an international interdisciplinary journal which publishes high quality original research, clinical trials and review articles, editorials, and commentaries relating to the etiopathogenesis, epidemiology, prevention, clinical features, diagnosis, treatment and management of patients with **neoplasms** in the **head** and **neck**.

Oral Oncology is of interest to head and neck surgeons, radiation and medical oncologists, maxillo-facial surgeons, oto-rhino-laryngologists, plastic surgeons, pathologists, scientists, oral medical specialists, special care dentists, dental care professionals, general dental practitioners, public health physicians, palliative care physicians, nurses, radiologists, radiographers, dieticians, occupational therapists, speech and language therapists, nutritionists, clinical and health psychologists and counselors, professionals in end of life care, as well as others interested in these fields.

Basic, translational, or clinical Research or Review papers of high quality and that make a contribution to new knowledge are invited on the following aspects of neoplasms arising in the head and neck (including lip, tongue, oral cavity, oropharynx, salivary glands, sinuses, nose, nasopharynx, larynx, skull base, thyroid, and craniofacial region, and the related hard and soft tissues and lymph nodes):

• **Etiopathogenesis**: natural history of cancer and pre-cancer; basic pathology, metastatic mechanisms; genetic changes; cellular and molecular changes; microorganisms; growth factors, adhesion and other molecules
• **Epidemiology**; risk factors; biomarkers; protective factors; geographic factors; prevention; screening and intervention
• Clinical features; **orofacial** effects of neoplasms at both local and distant sites; tumor staging and grading
• Diagnosis; **detection** of **cancer** and pre-cancer; cellular and molecular markers for diagnosis; advances in **imaging** and other functional diagnostic modalities for cancer and pre-cancer
• **Management** and **Prognosis**; clinical, cellular and molecular markers for prognosis; **treatment** options including surgical, lasers, photodynamic therapy, cryosurgery, micro- vascular and other forms of surgery, medical, radiotherapy, chemotherapy, immunotherapy, biological and gene therapy advances; molecular targets and new therapeutics (new cytotonics and molecular-targeted therapies); multimodality treatment; advances in reconstruction and rehabilitation, including flaps and grafts, alloplasty, bone and connective tissue biology; multidisciplinary teamwork in cancer care and **oral health care**.
• Quality of life issues; issues of consent; psychosocial aspects; patient and health professional information; patient involvement; psychological interventions, improving outcomes; the prevention,

GUIDE FOR AUTHORS

INTRODUCTION

Types of paper

Oral Oncology accepts the following article types for publication:

Editorial:

Authors who are considering submitting an editorial should contact the Editor-in-Chief with a brief outline of the proposed contribution before submission. Editorials are welcome on any topic; however, they may also be related to work previously published in Oral Oncology. **Editorials have no abstract and no keywords, and are usually restricted to 1500 words, up to 10 references and up to 2 tables or figures if not agreed otherwise with the Editor-in-Chief.** The Editor-in-Chief can be contacted at ooncology@elsevier.com.

Original Research Articles:

Original research articles present results of original epidemiology and public health, basic, clinical and/or translational (basic research with clinical applications) research. This article focuses on new data collected by the author(s) during the course of a epidemiology and public health research; basic investigation; clinical trial; or translational research, although other studies may be cited for support. Original research articles, which have not been published previously, except in a preliminary form, may be submitted as original full-length research papers. The article should contain the following sections: Title Page, Abstract, Conflict of Interest Statement, Introduction, Patients (or Materials) and Methods, Results, Discussion, and Conclusion. Mechanics: **Research articles should contain an abstract, a list of up to 10 keywords and have a limit of 3,500 words, 7 figures and/or tables, and 60 references.**

Review Articles:

Review articles that are topical and a critical assessment of any aspect of head and neck are welcome. Review articles collate, describe, and evaluate prior publications of important head and neck subjects, accompanied by critical analysis leading to rational conclusions. These Reviews should contain very little, if any, original data from an author's own study; however, such data can be used to support the overall thesis of the article. We also accept targeted mini-reviews that cover specific topics or therapies as well as meta-analyses. Mechanics: **Review articles should contain a short abstract stating the goal of the review, an introduction, discussion, and conclusion. Review articles can contain up to 5,000 words, 7 figures and/or tables, and 120 references.**

Perspectives:

Perspectives are more focused than reviews and seek to review a topic from a particular view or opinion. Perspectives should review a particular field to identify outstanding issues and/or challenges and propose new hypotheses or directions. A Perspective may highlight emerging science, controversial opinions, or issues within the field and seek to address these controversies. They may be accepted from a single individual or a team. Mechanics: **Perspectives should contain a short abstract stating the goal of the review, an introduction, discussion, and conclusion. Perspective articles are limited to 2000 words, 3 figures and/or tables, and 45 references.**

Letters to the Editor:

Letters to the Editor relating to published work in Oral Oncology or other topics of interest including unpublished original research are welcome. If accepted Letters are published online only. Mechanics: **Letters should not exceed 1,000 words in length and can contain up to 2 figures and/or tables.**

Types of paper

Special Issues:

If you have been invited to submit a manuscript for a Special Issue, please note that we recommend writing about 3,000 words and encourage diagrams, tables, and figures. There is no limit on references. **References in a special issue** - Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue. When submitting your manuscript, please be sure to select the correct Special Issue designation in the Peer Review System. **If you have any questions, please contact the inviting Editor**.

After reading the Guide for Authors, please visit our online submission system to submit your manuscript: http://ees.elsevier.com/oo.

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You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:
- E-mail address
- Full postal address

All necessary files have been uploaded:
Manuscript:
- Include keywords
- All figures (include relevant captions)
- All tables (including titles, description, footnotes)
- Ensure all figure and table citations in the text match the files provided
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Graphical Abstracts / Highlights files (where applicable)
Supplemental files (where applicable)

Further considerations
- Manuscript has been 'spell checked' and 'grammar checked'
- All references mentioned in the Reference List are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including the Internet)
- A competing interests statement is provided, even if the authors have no competing interests to declare
- Journal policies detailed in this guide have been reviewed
- Referee suggestions and contact details provided, based on journal requirements

For further information, visit our Support Center.

BEFORE YOU BEGIN

Ethics in publishing

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

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